

A LABORATORY ATLAS OF THE 13-MM. PIG EMBRYO

(Prefaced by younger stages of the chick embryo)

by

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THIRD EDITION

(Reprinted)

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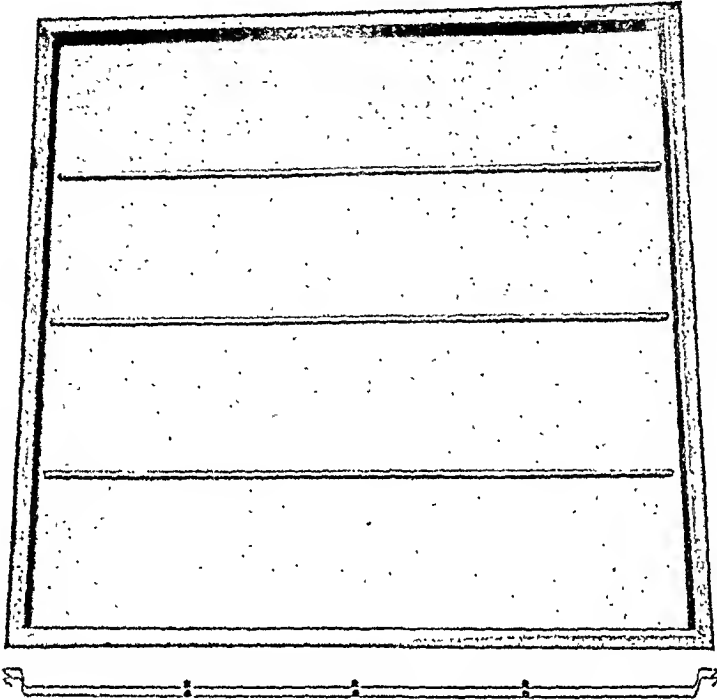
Since the primary object is mammalian organogeny, as little space as possible has been devoted to earlier stages in development. The eight sections of the 48-hour chick have been selected as those best adapted for making the transition between the 36-hour chick and the 13-mm. pig embryo. Blank pages have been provided throughout the book for drawings of such supplementary material as germ cells, germ-layer stages, histogenesis, dissections of older embryos, etc., to make the Atlas more adaptable to various types of courses given in zoological and anatomical departments.

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A MACROPHAGE-PROMOTING FACTOR (MPF) IN THE BLOOD OF RABBITS¹

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TWENTY-FIVE FIGURES

In the years following Harrison's demonstration of tissue culture technique as a useful tool for investigations in experimental morphology, workers were concerned with the selection of the most suitable nutrients for the maintenance of cells *in vitro*. In addition to Carrel's fundamental finding that embryonic extract was essential for the induction of high rates of cell division, studies were made on the effects of heterologous sera. Lambert and Hanes ('11a) found that homologous plasma was more favorable for the cultivation of mouse and rat tumors but that growth did occur if the plasma were interchanged. The production of satisfactory growth of rat sarcoma and of mouse carcinoma in guinea pig plasma as a culture medium led Lambert and Hanes ('11b) to conclude that the failure of successful heterospecific takes *in vivo* was not dependent upon food but on ". . . obscure and probably more complex factors." Goat plasma was found to be toxic for rat cells while pigeon plasma yielded good initial outgrowth but it was not found possible to maintain cells in this medium. Rabbit plasma was found less suitable than homologous plasma for the cultivation of tumors from other rodents. Walton ('14) corroborated these findings and stressed the importance of inhibitory and stimulating substances not necessarily dependent upon whether the plasma is autogeneous or homologous.

¹ Aided by grants from the U.S. Navy (N6onr-266) and from the American Cancer Society (CP-12).

Following these observations, studies on the effect of alien sera in tissue culture media were particularly directed toward the demonstration of immune body reactions. Of special interest was Bloom's ('27) demonstration of a macrophagic reaction in tissue culture in relation to an anti-erythrocytic antibody. Pigeon erythrocytes were rapidly phagocytosed *in vitro* by lung tissue of immunized rabbits while this effect was not often seen in control cultures using lung explants from non-immunized rabbits. Normal rabbit lung tissue showed macrophagic activity with the addition of either untreated or inactivated immune serum to the culture medium. Among the complicating factors reported by various workers in this field was the problem of animal individuality. Sera from some individuals were found to be decidedly toxic and thus obscured results.

In 1937 Carrel demonstrated that monocytes from different sources tended to assume the same appearance when cultivated in a given serum. Also, when leucocytes from a given animal were cultivated in sera of different individuals, a wide variety of cell modulations could be demonstrated. In discussing the peculiar individual qualities of sera, Parker ('38) made available excellent photographs of Carrel's unpublished experiments on blood cells in tissue cultures. When using delicate test objects such as members of the leucocytic series, investigators must consider not only the effect of heterologous sera but also the influence of sera from different individuals of the same species on the morphological and, presumably, the physiological response of cells.

Constitutional differences in human sera have been reported by Draper and his associates ('44, '45). Buffy coat cultures from individuals in 4 disease groups — poliomyelitis, peptic ulcer, coronary disease, and gall-bladder disease — were found to show differences in areolar size, form, and cellular content after 3½ hours *in vitro*.

Nantz and Blatt ('47) have suggested that buffy coat cultures might prove useful in the clinical evaluation of bacterial hypersensitivity. The protective action of anti-organ and of

normal rabbit sera has been recently demonstrated by Anigstein, Whitney and Beninson ('48a, '48b). Such sera injected into a limited skin area protect guinea pigs from infection when brain suspension containing the rickettsia of typhus or of spotted fever are injected within two hours into the infiltrated area. Subcutaneous injections of sera two inches from the point used for the introduction of the infective agents gave protection when diluted 1:5 with a testicular extract (Anigstein and Whitney, '48). The mechanism of this type of protection has not been explained.

In the course of studies dealing with the effect of adding reticulo-endothelial immune serum (REIS) to media in which embryonic chick spleen fragments were implanted, it was observed occasionally that great numbers of large phagocytic cells appeared in control cultures containing 25% normal rabbit serum (Pomerat and Anigstein, '45). The importance of developing methods for regulating the activity of the reticulo-endothelial system led us to make a systematic quest for a body in the plasma of rabbits which might favor the production of macrophages.

MATERIALS AND METHODS

The spleen from 18-day chick embryos was used in the majority of experiments as a test object for the production of macrophages. Under aseptic conditions spleens were cut into fragments approximately 1 mm square. One drop of a mixture of equal parts of embryonic extract derived from 7-day chicks and a serum to be tested was placed on a 7/8" sterile cover glass. A spleen fragment was transferred into this fluid. One drop of heparinized cockerel plasma was added quickly and the component materials were stirred to insure uniform mixing and clotting. Controls were prepared by substituting Tyrode's solution for the serum. Eight hanging-drop preparations were made for each serum in a particular series. Such tests were repeated on at least two different occasions to insure uniformity of results. More than 3000 hanging-drop

preparations were used in obtaining data for the present study.

Tissue cultures were examined in the living condition after incubation for 18 hours at 37.0°C. and a preliminary record was made of the character of the outgrowth. The preparations were then fixed in Helly-Zenker's fluid for one hour, washed several times with distilled water over a period of at least 6 hours and stained overnight with diluted Delafield's hematoxylin. After being washed in distilled water during the next day the preparations were counter-stained overnight with eosin-azure and then differentiated with 95% and absolute alcohol.

Serum to be tested for the presence of a factor capable of inducing macrophages in chick spleen cultures was obtained from 70 rabbits by collecting 15 ml of blood from the marginal ear vein. Blood collection from the majority of the other species studied was obtained by cardiac puncture with the exception of the larger animals in which the veins of the legs were used. Care was taken to assure asepsis and all sera were stored at 5°C. For fractionation procedures sera were lyophilized in a vacuum desiccator over phosphorus pentoxide. A "hi-vac" pump produced rapid freezing of the material. This apparatus achieved complete dehydration of 5 ml of serum within a few hours.

EXPERIMENTAL RESULTS

1. The phenomenon of rapid macrophage production in tissue cultures of chick embryo spleens by the serum of some "normal" rabbits

It is well known that tissue cultures of embryonic spleen are characterized by a rapid radial migration of the explanted cells. In the chick the highest rate of locomotion is achieved by myelocytes containing spherical or rod-shaped eosinophilic granules (cf. Rioch, '23). These cells are followed by lymphocytes and monocytes. By the end of the first day of incubation, fibroblasts usually become evident as pointed spikes of cells

emerging from the explants. Macrophages are not ordinarily numerous by the end of the first day and their phagocytic activity is insufficient to prevent the migration of myelocytes to the extreme periphery of the zone of migration. This can be seen in figures 3, 4, and 5, which show outgrowth from a spleen fragment in a hanging drop preparation from an 18-day chick embryo. The medium in this experiment consisted of 50% fowl plasma, 25% embryonic juice prepared from eggs incubated for 7 days, and 25% Tyrode's solution. This medium will be referred to as the *control* condition in results reported in this paper.

In experiments prepared with identical materials but with the substitution of heterologous sera for the Tyrode component, outgrowth from chick spleen fragments was not found to be notably different from the controls. Figures 6, 7, and 8 represent cell migration at various magnifications in a medium containing 25% human umbilical cord serum. A similar result was obtained by the substitution of sera from certain rabbits, but sera from other rabbits yielded an outgrowth pattern of notably different form and cellular components. Such a result is represented in figures 9, 10, and 11. With low power magnification (fig. 9), the total number of migrating cells can be compared with that found in the control (fig. 3). The outgrowth was divisible into an inner zone separated by a corona of densely packed cells from an outer area of migration. Examination of the outer zone (figs. 10 and 11) showed that it was not made up of myelocytes as in the control (figs. 4 and 5) and the cultures containing umbilical cord serum (figs. 7 and 8), but that it was characterized by a population of cells almost exclusively of the macrophagic type, the cytoplasm of these phagocytic elements being packed with nuclear and other debris. Cultures stained with hematoxylin-eosin-azure made possible the identification of ingested rod-shaped and spherical acidophilic granules (fig. 1). On the basis of experiments of this type it became clear that the blood serum of certain rabbits can induce the appearance of large numbers of macrophages in the peripheral outwandering of cells from

the spleen of 18-day chick embryos when cultivated at 37.0°C. for 18 hours. It became expedient to refer to a macrophage-promoting factor (MPF). The purpose of the present study is the description of this body and its mode of action.

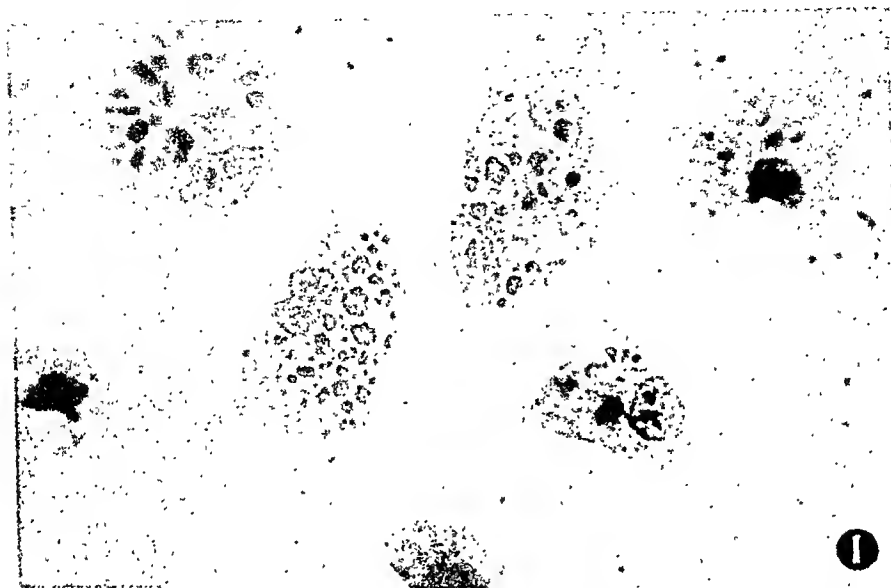


Fig. 1 Tissue culture of the spleen of an 18-day chick embryo showing the ingestion of myelocyte debris by macrophages which appeared within 24 hours of incubation. The medium had a final concentration of 25% rabbit serum containing a macrophage-promoting factor (MPF). $\times 735$.

2. Occurrence of MPF in rabbits and selection of test spleen

Initial experiments were conducted on a group of some 20 rabbits whose genetic history was not known. Results were confirmed on a group of 50 New Zealand albino stock line-bred animals. This herd had been line-bred for 32 years.² The macrophage-promoting capacity of each serum was evaluated according to the criteria described in table 1. On this basis 14% were 3+, 24% 2+, 30% 1+, 10% $-\pm$, and 28% showed no evidence of macrophage stimulation. No correla-

² Acknowledgment is made to Mr. and Mrs. Godfrey Adams of La Porte, Texas, for the use of these animals.

tion could be established with sex, pregnancy, high or low percentage of lymphocytes or any other characteristics in the differential count.

Spleens from chick embryos ranging in age from 10 days to hatching, newly hatched, and two-day chicks all showed the macrophage reaction when tested with serum containing the MPF. Spleen of 18-day chick embryos was used in routine tests. Aseptic technique can be easily maintained with such material.

TABLE 1

Criteria for rating macrophage reaction in chick spleen cultures

| AREA OF OUT-WANDERING CELLS | MYELOCYTES | MACROPHAGES | RATING |
|-----------------------------|-------------------------------------|--|--------|
| Like control | Like control | A few in intermediate zone | + |
| Slightly reduced | Slightly reduced in peripheral zone | Moderate number in both intermediate and peripheral zone | ++ |
| Moderately reduced | Few in peripheral zone | Many in peripheral zone | +++ |
| Markedly reduced | None in peripheral zone | Many in peripheral zone | ++++ |

In the early phases of this study spleen fragments from 10 to 12 chick embryos were "pooled" and used as explants for a given series of tests. Occasionally it was observed that a spleen fragment in the presence of a positive serum showed the macrophage reaction to a lesser extent than the other explants. In attempting to analyze this phenomenon 16 to 20 fragments derived from one spleen were used to test two or three sera. It was found that the spleens from nearly all embryos gave consistently positive results. Occasionally the explants derived from one spleen gave only weakly positive (or even negative) results when tested with sera which had shown a strong tendency to produce macrophages in cultures

of "pooled" fragments. This suggested that the MPF may bear a relationship to some individual factor in the test material. A systematic, quantitative study of this problem with special reference to "blood types" is now in progress.

3. Constancy of MPF in individual rabbits

Once rabbits were found to have sera clearly negative or positive for the MPF, they were segregated for continued observation. It was soon noted that the factor was not constantly present or absent. Results showing variation over a

TABLE 2

Variations in the macrophage reactions induced in spleen explants of 18-day embryos by sera obtained from the same rabbits at different times

| RABBIT NUMBER | DATE BLED | MACROPHAGE REACTION |
|---------------|-----------|---------------------|
| 95 | 11- 2-47 | ± |
| 95 | 1-20-48 | — |
| 95 | 1-31-48 | ++++ |
| 95 | 3-25-48 | ++++ |
| 95 | 4-20-48 | +++ |
| 2 | 10- 4-47 | — |
| 2 | 1-12-48 | — |
| 2 | 3-25-48 | ++++ |
| 2 | 4-20-48 | +++ |
| 117 | 2-11-48 | — |
| 117 | 3-25-48 | +++ |

period of several months are recorded in table 2. No factor in differential counts was found to be correlated with the intensity of the macrophage reaction produced by the serum. Inadvertently, animal no. 95 was used on 3/19/48 for an anterior chamber implantation of the Rous chicken sarcoma. This grew rapidly with considerable attending abscess formation. At the time of killing on 5/8/48, the serum of this animal almost completely inhibited the migration of chick spleen cells.

4. *Macrophage promotion in relation to time of incubation*

Control and MPF-treated cultures were fixed at two-hour intervals. Typical results are represented in figures 12 to 19. In Tyrode controls a very large number of migrating cells were seen under low power to form a dense corona around the explant after two hours of incubation. The grouping of these cells was still evident at 4 hours (fig. 12) but by 8 hours (fig. 13) they had extended to produce a relatively uniformly scattered circle around the explant. The limits of migration were reached between 8 to 10 hours.

Cultures treated with MPF serum showed, under low power, outgrowth at two and 4 hours (fig. 14) which was scant and without concentration in the form of a corona, but, between the 6th and 14th hours (fig. 15), the cultures were characterized by a dense circumscribed sheet of migrating cells showing marked aggregation at the periphery and covering approximately half the distance found for the corresponding controls (fig. 13).

Tyrode control cultures from the 2nd (fig. 16) to the 20th hours (fig. 17) showed under high power that the periphery of the zone of migration consisted of eosinophilic myelocytes. These were usually fairly rounded but occasionally the granules were in elongated pseudopodia.

Peripheral cells in cultures treated with MPF serum observed at high power between the 2nd and 6th hours resembled the eosinophilic myelocytes seen in corresponding controls. By the 8th hour (fig. 18) such cells appeared to be disintegrating. The nuclei were occasionally pycnotic and the cytoplasm swollen with resulting scattering of the eosinophilic granules. Cultures incubated for 10 hours showed cells which were interpreted as representing young macrophages (polyblasts) containing small amounts of amorphous material. At 12 and 14 hours (fig. 19) phagocytosis of disintegrated myelocytes seemed to be intensified. At 20 hours the peripheral field was made up almost exclusively of large macro-

phages containing in their cytoplasm numerous phagocytosed nuclear remnants and a variety of cellular granules including eosinophilic spheres and rodlets. It appears, therefore, that the disintegration of the myelocytes precedes the formation of the macrophages.

TABLE 3

*Macrophage reaction was not correlated with occurrence of Forssman antigen
(Sera used at 1:4 in culture medium)*

| FORSSMAN PRESENT | FORSSMAN ABSENT | MACROPHAGE REACTION |
|------------------|--------------------------|---------------------|
| Toad | | negative |
| | Frog | negative |
| Chicken | | negative |
| | Goose | negative ? |
| Goat | | negative |
| Sheep | | negative |
| | Cow | negative |
| | Pig (γ globulin) | negative |
| Mouse | | negative |
| | Rat | negative |
| | Rabbit | positive |
| Cat | | negative |
| Man, group A | | negative |
| | Man | negative |

Other sera which produced negative results: turkey, guinea hen, duck, human umbilical cord serum.

5. Quest for the MPF in other species and in relation to the various antibodies

Table 3 presents a list of animals known to have or to be free from the Forssman antigen (Boyd, '47). Of the species listed only rabbit serum was found to have the MPF. Goose serum was found negative, but when goose plasma was substituted for cockerel plasma considerable numbers of macrophages accumulated at the periphery of the cultures. They were rated at 2+ for the MPF reaction. Porcine gamma globulin at high concentration did not produce the reaction. Sera from at least 5 animals were tried for each species reported.

Current studies on anti-organ sera (Anigstein, Whitney, Pomerat and Orr, '47) provided material showing that rises in hemolytic titre or the development of anti-organ titres as high as 1:1500 measured by complement fixation were not associated with the appearance of the MPF (table 4). Anti-organ sera produce a different effect on embryonic chick spleen in tissue culture. Limitation of outgrowth has been found to be correlated with the strength of the anti-organ antibody as measured by complement-fixation (Pomerat and

TABLE 4

Macrophage reaction not induced even by increasing hemolytic or complement-fixation titre of rabbits (by anti-organ immunization)

| RABBIT NUMBER | CONDITION | HEMOLYTIC TITRE | C.F. TITRE |
|---------------|--------------------------------|-----------------|------------|
| 93 | normal | 1:30 | — |
| 93 | after G.P. ¹ spleen | 1:5000 | 1:1500 |
| 94 | normal | 1:30 | — |
| 94 | after G.P. spleen | 1:5000 | 1:1000 |
| 96 | normal | 1:250 | — |
| 96 | after chick brain | 1:500 | 1:600 |
| 97 | normal | 1:80 | — |
| 97 | after chick brain | 1:2500 | 1:300 |
| 98 | normal | 1:20 | — |
| 98 | after G.P. kidney | 1:2500 | 1:600 |

¹ G.P. = guinea pig.

Anigstein, '45): Clumping of cells, especially at the periphery, and coronal aggregations are common, but the induction of a macrophage reaction has never been correlated with sera having high anti-organ titres.

Normal and immune sera have been shown to influence the properties of the cell surface and to produce phagocytic effects which are strikingly parallel when studied with serial dilutions of serum. In classical papers, Lucké, McCutcheon, Strumia, and Mudd ('29, '33) have proposed that the relationships between particles, phagocytes, and the media are altered

by serum protein in such a manner as to favor phagocytosis. Opsonions and tropins are thus considered to operate by altering "protein-wetting" and by other physico-chemical effects.

6. Attempts to reproduce the MPF phenomenon with various drugs

According to Moon ('38) and to Jansco ('47), histamine can influence the reticulo-endothelial system by inducing the transformation of resting endothelial cells into active phagocytes. Histamine incorporated in culture medium at concen-

TABLE 5

Macrophages were not induced in these tests (24 hrs.)

| HISTAMINE ACID PHOSPHATE (2.75 MG/ML) | | | |
|--|---|--------------|---|
| 1:4,000 | } | like control | |
| 1:8,000 | | | |
| HISTAMINE PHOSPHATE (1:1,000 AMPOULES) | | | |
| 1:4,000 | } | 1:160,000 | } |
| 1:8,000 | | 1:320,000 | |
| 1:10,000 | | 1:640,000 | |
| 1:20,000 | | 1:1,280,000 | |
| 1:40,000 | | 1:2,560,000 | |
| 1:80,000 | | | |
| like control | | like control | |

trations ranging from 1:4000 to 1:2,560,000 failed to produce the MPF reaction (table 5). Cells migrating from spleen explants appeared uninjured after 24 hours incubation in a medium which contained histamine at a concentration of 1:4000.

Following upon the work of von Möllendorff ('32) and of Thomas ('38), Chevremont ('43, '47) has made important contributions dealing with the experimental transformation of various cell types into macrophages. When choline chloride at a final concentration of M/250 to M/300 was added to medium used for the culture of skeletal muscle it produced a threefold increase in the number of macrophages as com-

pared with untreated controls. He also found that various quaternary ammonium compounds favored the appearance of macrophages in cultures. With our techniques, however, critical concentrations of choline chloride, mecholyl chloride, tetraethanol ammonium hydroxide, and etamon tested on chick spleen fragments were not found to produce macrophages within the 18-hour period utilized in our definition of the MPF effect (table 6).

TABLE 6

Macrophage reaction not induced in these tests (24 hrs)

| CHOLINE CHLORIDE | | TETRAETHANOL AMMONIUM HYDROXIDE | |
|-------------------|-------------------------|---------------------------------|-------------------------------|
| M/13.8 | like control | M/16.3 | few rounded cells |
| M/34.5 | | M/81.6 | rounded cells and clumping |
| M/69.0 | | M/163.2 | |
| M/138.0 | | M/244.8 | |
| M/276.0 | | M/408.0 | like control |
| M/345.0 | | M/489.6 | |
| | | M/652.8 | |
| MECHOLYL CHLORIDE | | ETAMON | |
| M/17.0 | restricted outgrowth | M/7 | only a few |
| M/84.8 | | M/33 | rounded cells |
| M/254.4 | | M/170 | some injury |
| M/424.0 | like control | M/200 | |
| M/848.0 | | | |
| M/1272.0 | | | |

Heilman ('45) has reported that macrophages migrating from mammalian lymph node appeared to be stimulated by 11-dehydro-17-hydroxycorticosterone (Kendall's compound E) at 2.5 to 60 mg per milliliter. She obtained the same effect with 11-dehydrocorticosterone (Kendall's compound A) at concentrations ranging from 5 to 60 mg per milliliter. The macrophage reaction was not induced by the addition of 50 mg of 11-dehydro-17-hydroxycorticosterone to the medium used in our standard test.

7. Fractionation

The procedure used in the separation of the active factor in macrophage-promoting serum may be seen in figure 2. Actual results obtained with tissue culture tests are shown in figures 20 to 25. An active serum (no. 95) was lyophilized and restored to its original volume. It proved to be as potent in its MPF activity as the original material. When 5 ml of such

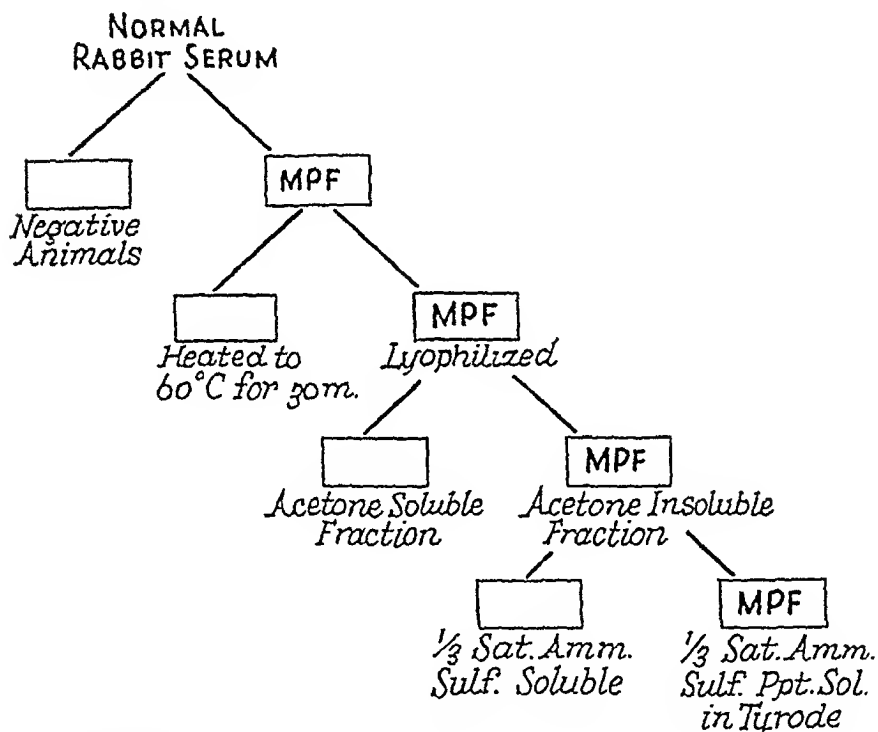


Fig. 2 Procedure used in the separation of the active factor in macrophage-promoting serum.

serum was lyophilized and restored with only 2 ml of distilled water (2½ times the original concentration) and tested in the usual way, it showed a very powerful macrophage effect. At low power (fig. 20) cultures containing 25% of this restored serum were observed to be characterized by marked limitation of outgrowth with numerous large cells. At high power (fig. 21) the peripheral elements were found to be macro-

phages filled with a large amount of cellular debris. This indicates that freeze-drying had not impaired the macrophage-promoting capacity of the serum. The factor was found present in fractions insoluble in absolute ethyl and methyl alcohol.

The lyophilized material was extracted with acetone at 3°C. for 24 hours. The volume of acetone was 10 times the original volume of serum. The acetone-soluble material was dried *in vacuo* and the dry matter was resuspended in Tyrode. For each milliliter of original serum, 0.25 ml of Tyrode was used to resuspend the acetone-soluble material. Examination at low and high power (figs. 22 and 23) of test cultures showed outgrowth resembling that obtained in Tyrode controls. Thus the acetone-soluble fraction does not contain this factor.

The acetone-insoluble fraction of serum no. 95 restored to 2½ times its original concentration influenced cultures in the same manner as the unfractionated serum (fig. 24). At high power (fig. 25) peripheral cells were seen to be very large macrophages showing active phagocytosis.

Two volumes of a serum proven to contain a large amount of the MPF was mixed with one volume of saturated ammonium sulfate. After standing overnight at 3°C., the solution was centrifuged, the precipitate washed in a small volume of Tyrode, and dialyzed under sterile conditions against Tyrode at 3°C. for about 24 hours. The non-dialyzable fraction was tested in tissue culture and was found to have retained the MPF. The supernatant was placed in the dialyzing bag for ultrafiltration under pressure. In this way, the supernatant was reduced to about one-fifth of its original volume and then reconstituted by the addition of sterile Tyrode. This fraction proved inhibitory to spleen cells in initial tests, possibly due to the presence of small traces of ammonium sulfate. Further experiments showed that when this fraction was completely freed from ammonium sulfate by dialysis for 72 hours at 3°C. against Tyrode, results were similar to those obtained in the controls. This fraction, therefore, did not contain the MPF.

DISCUSSION

The phenomena considered in this paper involve the production under the influence of "normal" rabbit serum of large numbers of macrophages which entirely remove all outwandering myelocytic elements in tissue cultures of chick spleen fragments. While serial sections of the explants and differential counts of cell populations in culture have not yet been completed, careful examination of the outgrowth strongly suggests that the increase in macrophages is probably due to the transformation of reticulum cells or monocytic elements. Mitoses are not commonly seen in the zone of outwandering cells. The possibility that macrophages are produced by a process of nuclear budding or meromitosis as described by Thomas ('38) requires further study. It is well known that the form of cells varies under conditions of growth on the surface of the plasma coagulum in contrast to that seen when they are migrating within it. Macrophages tend to spread out and develop an "epithelioid" form when in direct contact with the cover glass especially in the presence of a pool of liquefied medium. Weiss ('45) has referred to a series of modifications in the form of Schwann cells from long slender spindles to large round macrophages as an illustration of cellular "modulations."

It is believed that the total number of outwandering myelocytes in MPF treated cultures is probably approximately the same as that found in the controls. Observations on cultures fixed at various times lead to the conclusion that the myelocytic elements are injured within about 8 hours. Marked increase in the total number and in the size of macrophages soon follows. The ultimate disappearance of practically all the myelocytes in the zone of migration can be accounted for by the enormous amount of ingested cellular debris found in the macrophages. The round and rod-shaped acidophilic granules of the myelocytes can be identified readily in the phagocytes with the aid of the hematoxylin-eosin-azure stain. Whether the production of macrophages is merely the result

of a primary injury to the myelocytes requires further elucidation.

In a series of fundamental papers dealing with the dynamics of inflammation, Menkin ('40, '46, '48) has described 5 bodies associated with experimentally induced pleural exudates. Some of the properties of these factors are summarized in table 7. The question arose as to whether the MPF was identical with one of these fractions or whether it was an additional

TABLE 7

Factors in inflammatory exudate as reported by Menkin

| FACTORS | ROLE | KEY CHARACTERISTICS |
|---------------------------------------|--|---|
| 1 Leucotaxin | Increases capillary permeability—local migration of leucocytes | THERMOSTABLE Soluble in alcohol |
| 2 Leucocytosis promoting factor (LPF) | Originates in inflamed area to produce leucocytosis. Stimulates granulopoiesis | THERMOLABILE ppt. at $\frac{1}{2}$ saturation with amm. sulf. (α_1 and α_2 globulins) |
| 3 Necrosin | Produces inflammation — associated with drop in pH of exudates | THERMOLABILE A euglobulin also in blood serum |
| 4 Pyrexin (pyrogenic factor) | Probably acts on heat-regulating center of hypothalamus | THERMOSTABLE Separated from euglobulin fraction by differential solubility |
| 5 Leucopenic factor | By trapping leucocytes in lungs, liver and spleen | THERMOSTABLE A polypeptide |

factor which may possibly be associated with inflammatory reaction. The three thermostable factors in Menkin's scheme, leucotaxin, pyrexin, and the leucopenic factor, can be ruled out since MPF is thermolabile. The leucocytosis-promoting factor (LPF) can also be excluded since it is not precipitated by 1/3 saturated ammonium sulfate. From Menkin's scheme of fractionation (Menkin, '46, p. 385) it would appear that

necrosin is present in the insoluble residue which is formed after prolonged dialysis of the ammonium sulfate precipitate. The macrophage-promoting factor, however, remains in solution under these conditions. It appears, therefore, that the macrophage factor is not identical with any of the fractions studied by Menkin. However, *in vivo* experiments are in progress to provide further evidence regarding these conclusions.

SUMMARY

The present study is concerned with the capacity of blood serum from "normal" rabbits to promote the formation of large numbers of macrophages in explants of spleen from 18-day chick embryos. When incubated at 37.0°C. for 18 hours, such cultures show that all outwandering myelocytes have been phagocytosed. Evidence is presented to show that a macrophage-promoting factor (MPF) which is present in varying degrees in the blood of rabbits is thermolabile, insoluble in absolute alcohol or acetone, and soluble in Tyrode after precipitation in 1/3 saturated ammonium sulfate. Under the test conditions described, it was not found possible to reproduce the macrophagic reaction *in vitro* by the addition to the medium of sera from animals known to have or to be free from the Forssman antigen or by anti-organ sera with high or low complement-fixation or hemolytic titres. Negative results were also obtained with various concentrations of histamine, 17-hydroxy-11-dehydrocorticosterone, choline, or various quaternary ammonium compounds. It is believed that the macrophage-promoting factor (MPF) is not identical with leucotaxin, the leucoctosis-promoting factor, necrosin, pyrexin, or the leucopenic factor described by Menkin.

LITERATURE CITED

- ANGSTEIN, L., AND D. WHITNEY 1948 The inhibitory effect of anti-organ sera on experimental typhus and Rocky Mountain spotted fever in presence of testicular extract. *Tex. Rep. on Biol. and Med.*, 6: 379-384.
- ANGSTEIN, L., D. WHITNEY AND J. BENINSON 1948a Inhibition of typhus and spotted fever by intradermal inoculation of anti-organ or certain normal sera. *Proc. Soc. Exp. Biol. and Med.*, 67: 73-74.

- ANIGSTEIN, L., D. WHITNEY AND J. BENINSON 1948b Inhibition of typhus and spotted fever in guinea pigs by intradermal inoculation of anti-organ sera and of certain normal sera. *Tex. Rep. on Biol. and Med.*, 6: 87-96.
- ANIGSTEIN, L., D. M. WHITNEY, C. M. POMERAT AND M. F. ORR 1947 Reticulo-endothelial immune serum (REIS). VI. Production of potent serum by anamnestic reaction. *Proc. Soc. Exp. Biol. and Med.*, 64: 279-280.
- BLOOM, WILLIAM 1927 Immune reactions in tissue culture. I. Reaction of lungs from normal and immunized rabbits to pigeon erythrocytes. *Arch. Path. and Lab. Med.*, 3: 608-628.
- BOYD, WILLIAM 1947 *Fundamentals of Immunology*. Interscience Press, Inc., N. Y.
- CHEVREMONT, M. 1943 Recherches sur la production experimentale de la transformation histiocytaire dans les cultures in vitro. *Arch. de Biol.*, 54: 377-407.
- 1947 La transformation histiocytaire et son determinisme. *International Cytological Congress, Stockholm, Sweden* (unpublished).
- DRAPER, G., H. J. RANSEY AND C. W. DUPERTUIS 1944 Variation in behavior of buffy coat cultures among individuals of different constitution types. *J. Clin. Invest.*, 23: 864-874.
- DRAPER, G., C. PIERCE AND C. W. DUPERTUIS 1945 The relationship between cells and plasma in cultures of the buffy coat from human blood. *Am. J. Med. Sci.*, 210: 738-745.
- HEILMAN, DOROTHY H. 1944 The effect of 11-dehydro-17-hydroxycorticosterone and 11-dehydrocorticosterone on migration of macrophages in tissue culture. *Proc. Staff Meet. Mayo Clinic*, 20: 18, 318-320.
- JANSKO, MIKLOS 1947 Histamine as a physiological activator of the reticulo-endothelial system. *Nature*, 160: 227-228.
- LAMBERT, R. A., AND F. M. HANES 1911a A comparison of the growth of sarcoma and carcinoma cultivated in vitro. *Proc. Soc. Exp. Biol. and Med.*, 8: 59-60.
- 1911b The cultivation of tissue in plasma from alien species. *J. Exp. Med.*, 14: 129-138.
- LUCKÉ, B., M. STRUMIA, S. MUDD, M. McCUTCHEON AND E. B. H. MUDD 1933 On the comparative phagocytic activity of macrophages and polymorphonuclear leukocytes. The essential similarity of tropin action with respect to the two types of phagocyte. *J. Immunol.*, 24: 455-492.
- MENKIN, VALY 1940 *Dynamics of Inflammation*. The Macmillan Co., N. Y.
- 1946 Chemical factors and their role in inflammation. *Arch. of Path.*, 41: 376-387.
- 1948 The change in the leucocyte formula by the leucocytosis-promoting factor of exudates in experimental leucemia. *Science*, 107: 546-547.
- VON MÖLLENDORFF, M. 1932 Phagozytoseversuche mit fibrozyten. *Z. Zellforsch.*, 15: 274-283.
- MOON, V. 1938 *Shock and related capillary phenomena*. Oxford Univ. Press, N. Y.

- MUDD, S., B. LUCKÉ, M. McCUTCHEON AND M. STRUMIA 1929 On the mechanism of opsonin and bacteriotropin action. *J. Exp. Med.*, 49: 779-795.
- NANTZ, FRANK A., AND HERMANN BLATT 1947 The application of a tissue culture technique in the clinical evaluation of bacterial hypersensitivity. *Ann. of Allergy*, 5: 554-557.
- PARKER, RAYMOND C. 1938 *Methods of Tissue Culture*. Paul B. Hoeber, Inc., Med. Book Dept. of Harper and Bros., N. Y.
- POMERAT, C. M., AND L. ANIGSTEIN 1945 Reticulo-endothelial immune serum (REIS): I. Its action on spleen *in vitro*. *Tex. Rep. on Biol. and Med.*, 3: 1, 122-141.
- RIOCH, DAVID 1923 The morphology and behavior of the migratory cells in tissue cultures of the chick's spleen. *Anat. Rec.*, 25: 41-57.
- THOMAS, J. A. 1938 Recherches sur les transformations la multiplication et la specificite des cellules hors de l'organisme. *Ann. Sci. Nat. Zool.*, 1: 210-579.
- WALTON, A. J. 1914 Variations in the growth of adult mammalian tissue in autogenous and homogenous plasma. *Proc. Roy. Soc. London, Series B*, 87: 452-461.
- WEISS, PAUL, AND HSI WANG 1945 Transformation of adult Schwann cells into macrophages. *Proc. Soc. Exp. Biol. and Med.*, 58: 273-275.

PLATES

PLATE 1

EXPLANATION OF FIGURES

Outwandering of cells from spleen explants of 18-day chick embryos. Figures 3, 4, and 5 at $\times 16$; 6, 7, and 8 at $\times 75$; 9, 10, and 11 at $\times 450$.

3, 4, and 5 From a control culture containing a final concentration of 2.5% Tyrode.

6, 7, and 8 From a culture containing a final concentration of 2.5% human umbilical cord serum.

9, 10, and 11 From a culture with a final concentration of 2.5% rabbit serum containing a macrophage-promoting factor (MPF).

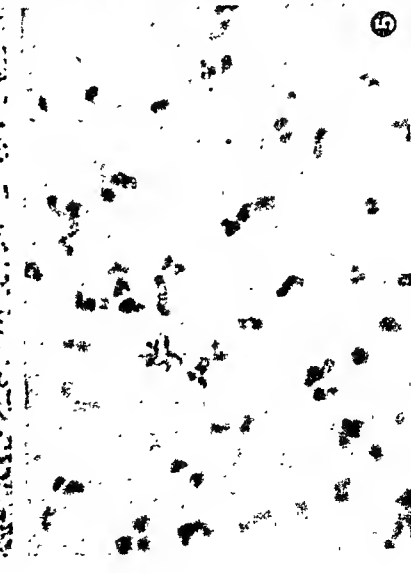
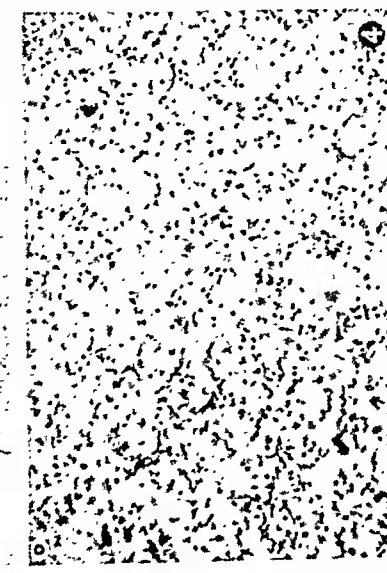
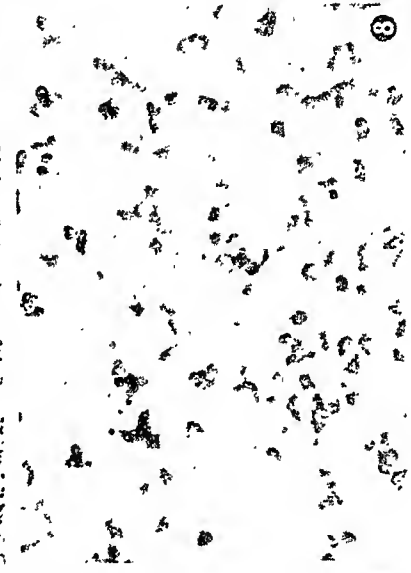
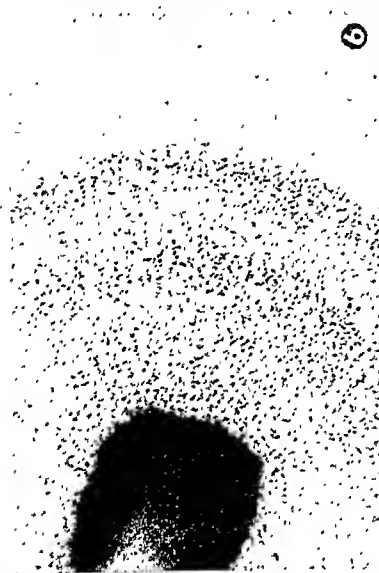
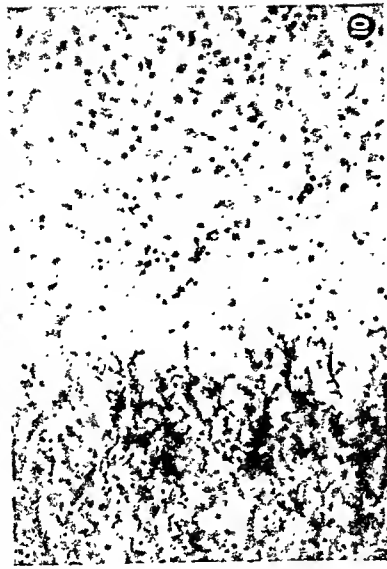
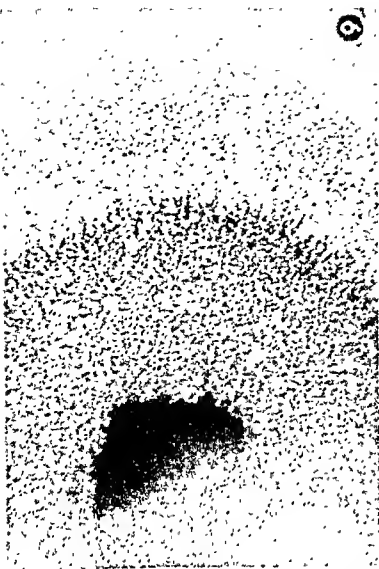


PLATE 2

EXPLANATION OF FIGURES

Ontwandering of cells from spleen explants of 18-day chick embryos. Figures 12, 13, 14, and 15 at $\times 24$; 16, 17, 18, and 19 taken at margin of outgrowth at $\times 255$.

12 Tyrode control; 4 hours incubation.

13 Tyrode control; 8 hours incubation.

14 Outgrowth in the presence of a macrophage-promoting factor (MPF) from an untreated rabbit; 4 hours incubation.

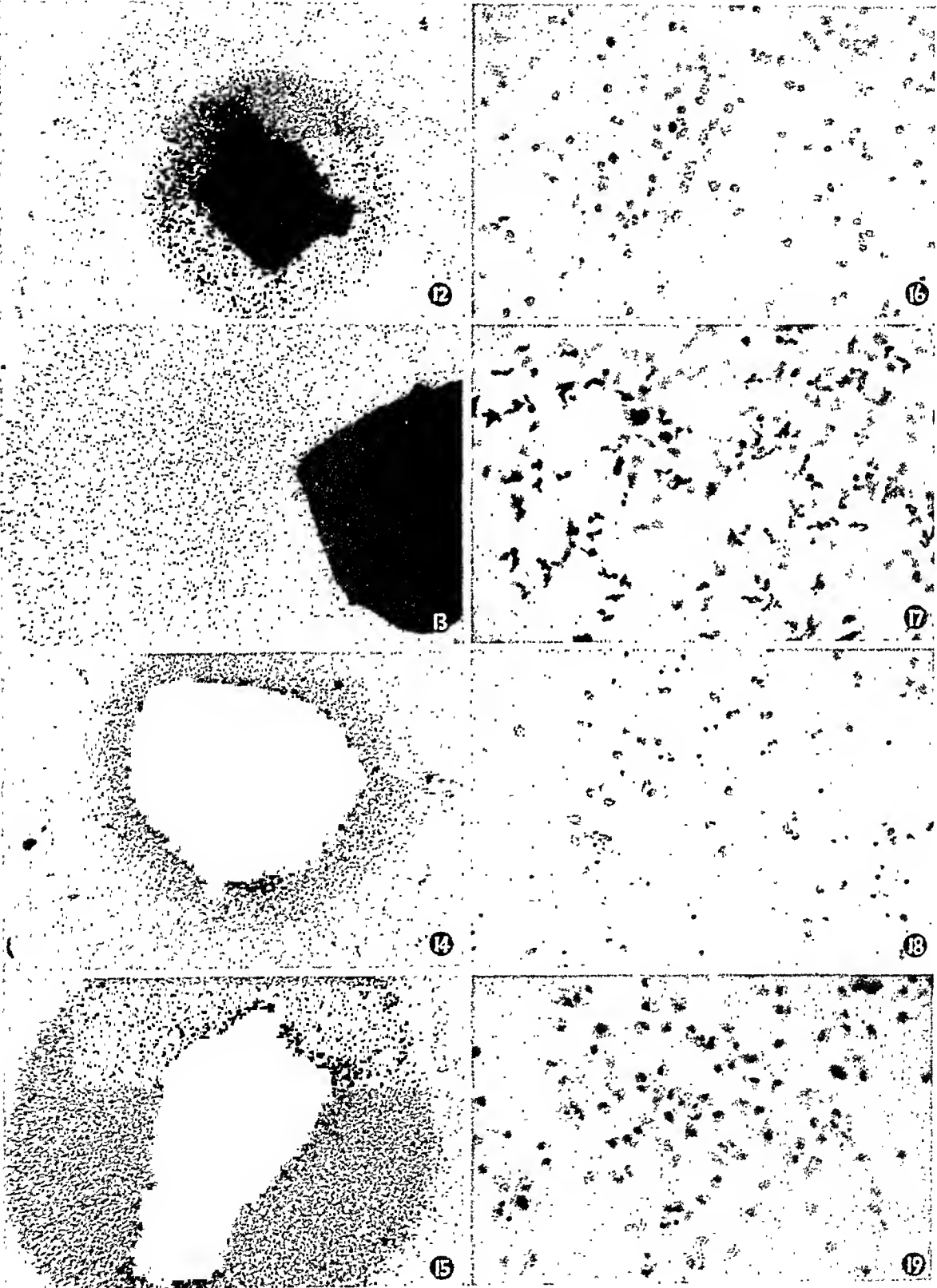
15 Culture treated with MPF; 8 hours incubation.

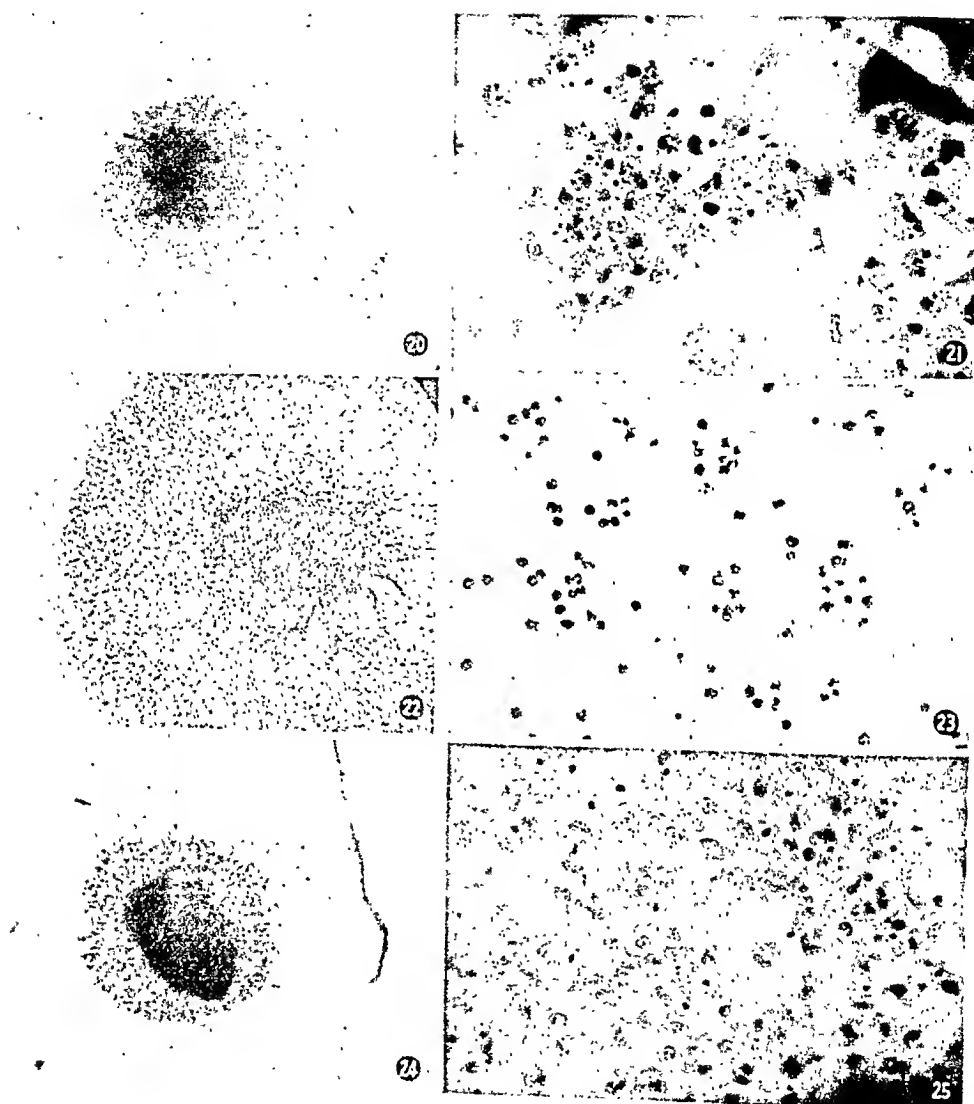
16 Tyrode control; typical myelocytes at margin of outwandering cells; 2 hours incubation.

17 Tyrode control; myelocytes showing pseudopodia; 20 hours incubation.

18 Culture treated with MPF; myelocytes showing swelling and scattering of granules; 8 hours incubation.

19 Culture treated with MPF; cell population at margin of outgrowth composed almost exclusively of medium-sized macrophages; phagocytosis of myelocytes almost complete; 14 hours incubation.





Outwandering of cells from spleen explants of 18-day chick embryos. The medium contained 25% of the test material. Figures 20, 21, and 22 at approximately $\times 12$; 23, 24, and 25 at $\times 255$.

20 and 21 From a culture containing serum from rabbit no. 95 showing a marked reaction due to the macrophage-promoting factor (MPF).

22 and 23 From a culture containing the acetone-soluble fraction of rabbit serum no. 95.

24 and 25 Containing the acetone-insoluble fraction of rabbit serum no. 95 showing that the macrophage-promoting factor is contained in this separation.

OBSERVATIONS ON THE BLOOD SUPPLY OF GROWING ANTLERS

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TWENTY-ONE FIGURES

A description of the blood supply of growing antlers, together with a comprehensive survey of the available literature, was published in 1942 by one of us (Wislocki). Since that time, continued observation and experimentation on a group of Virginia deer (*Odocoileus virginianus borealis*) and several Sika deer (*Cervus nipon*) have provided further information on this topic.¹ In the present paper an account of these recent investigations is given.

MATERIAL AND METHODS

The material for the present study was derived from (1) the heads of two normal deer injected at death with India ink, (2) the heads of two deer similarly injected whose antlers had been ligated some weeks before death, and (3) the antlers of one deer, ligated but not injected with ink. This material was supplemented by antemortem and postmortem observations on antlers which had been injured or cut into surgically. One of the two normal deer was a Sika deer, whereas the 4 other animals referred to were Virginia deer.

¹ The Virginia deer antlers used in this investigation were obtained from the Wildlife Research Center, Delmar, New York, under an agreement with the Conservation Department of the State of New York. We are greatly indebted to the administrative officers and scientific personnel of that organization for their friendly aid and cooperation. The Sika deer antlers were obtained through the kindness of Mr. James Draper of Canton, Massachusetts, to whom we wish to express our appreciation.

The blood vessels of the antlers were injected by the following methods. In the case of the first head, a mixture consisting of equal parts of Higgins' waterproof India ink and water was injected through the common carotid arteries. In the other three heads, the injections were made into the superficial temporal arteries proximal to their major branches. A large metal syringe holding 200 cm³ was coupled by rubber tubing to a glass cannula tied into the artery. About 400 cm³ of the ink mixture were injected into each artery under as much pressure as it was possible to exert by hand. After completion of the injection, the antlers and their pedicles were removed, cut into suitable pieces, and placed in 10% neutral formalin for 4 or 5 days. Some of these pieces were subsequently decalcified in 4% trichloroacetic acid, cleared by the Spalteholz method and cut into free-hand sections from 1 to 3 mm thick. A few pieces were embedded in celloidin so that thinner sections from 20 to 200 μ in thickness could be cut.

Uninjected pieces of growing antler tip were placed in several special fixatives in order to carry out a variety of staining procedures. These blocks were embedded in paraffin and were cut into 10 μ sections without preliminary decalcification. Sections from a block placed in Zenker's fluid were stained either with methylene blue and eosin or by the azan technique (figs. 7 and 16). Other sections, after fixation in cold acetone, were stained by Bodian's protargol method (fig. 17). Still other sections, fixed in Zenker's fluid or 10% formalin, were impregnated with silver by Pap's method for reticulum (fig. 15).

Ligatures were placed upon one or both of the growing antlers of three of the deer mentioned above. Insulated copper bell wire or heavy silk was used, twisted or tied tightly enough to sink deeply into the velvet without cutting it. From one to 9 weeks later the animals were killed and the antlers were injected with ink.

Occasional observations on antlers which had been injured or cut into surgically provided additional information regarding the blood supply and circulation. The observations on

antlers subjected to surgery were made on animals anesthetized with pentobarbitol. Shed antlers and the heads of deer killed accidentally provided a further source of material. No essential differences were observed between the blood vessels of the antlers of Virginia deer and Sika deer, and consequently the two will not be described separately.

OBSERVATIONS

The antlers of deer are deciduous structures which are annually renewed. For the Virginia deer in the northeastern part of the United States, antler growth begins in late April or early May and continues until late August. The investing skin or "velvet" is shed in September. The mature antlers, consisting of bare, dead bone, remain in place, firmly attached, until mid-winter when they are shed.

Antlers grow by the addition of new material at the extremity of the beam and at the tips of the tines as they arise. A growing tip moves away from the antler base depositing a column of bone which, once laid down, does not increase appreciably in diameter as it matures. Nearly all stages of growth and maturation can be seen in a single growing antler; the bone nearest the antler base is the oldest and most mature, whereas that at the tips is in process of formation. The topography of the tip of an ossifying antler is illustrated in figure 7; the germinal (G), preosseous (P) and osseous (O) zones are recognizable beneath the velvet (V).

When the antler first develops in May it is composed of delicate spongy bone. In June, July and August it gradually undergoes internal reconstruction. The peripheral or cortical part is converted into dense, compact bone having primary Haversian systems, while the cancellous interior comes to be composed of fewer and coarser spicules of spongy bone enclosing relatively wide marrow spaces. Secondary and tertiary Haversian systems and the resulting interstitial lamellae which characterize skeletal bone are lacking. Because the antler is laid down in its entire width from the outset,

circumferential lamellae are not formed. Details of these changes are presented by Wislocki ('42).

The reorganization of bone at the base differs in some respects from that which occurs in the rest of the antler. For 2 or 3 cm above the junction of the antler with its pedicle the bone becomes relatively hard and exceedingly dense without any central cancellous structure whatsoever. In the zone of union of base and pedicle, the Haversian systems interweave irregularly, whereas they run rather parallel to one another in the cortical bone of the rest of the antler. The impression is gained that this arrangement may lend particular strength and hardness to the base of the antler. The burrs of the corona are also composed of irregularly arranged bony lamellae.

It should be noted that, while growth is occurring at the lengthening tips of the antlers by the laying down of spongy bone, the internal reorganization described above is taking place progressively from the tips toward the base of the antler, the latter being composed of the oldest bone. In late July and August, as the antlers attain their ultimate length and growth nears completion, the tapering ends of the tines also become converted into densely calcified bone. The more distal tines complete their growth later than those located near the antler base.

The blood supply as revealed by India ink injections. The blood supply of the antler was investigated in 4 heads in which the vessels had been injected with India ink.

A composite of the typical vascular elements to be seen in injected and cleared specimens is shown diagrammatically in figure 1. Here a growing antler is represented greatly compressed in length to bring into close approximation successive regions from the rapidly growing germinal cap and preosseous zone of the tip (I and III) to the compact Haversian bone of the base of the antler (VI). The intermediate regions are composed of spongy bone which is of uniformly cancellous structure near the tip, but becomes denser peripherally (IV) and more coarsely trabeculated internally (V), as the pedicle

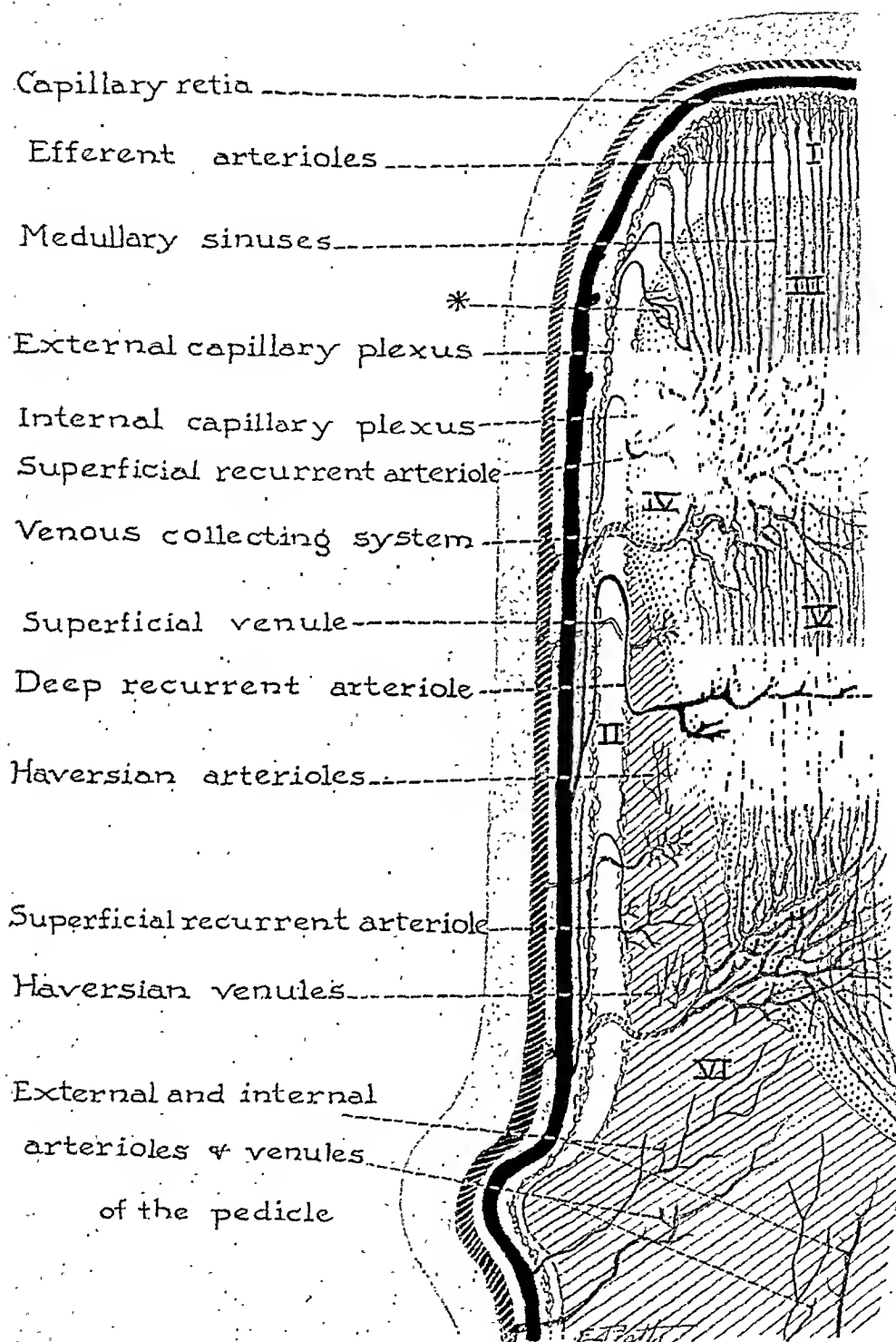


Fig. 1 Composite diagram showing the vascular elements of a growing antler. I, germinal cap; II, fibrocellular periosteal layer; III, proosseous zone; IV, peripheral cancellous bone; V, core of coarsely trabeculated bone with wide medullary spaces; VI, compact Haversian bone.

is approached. Across the base (VI) the internal as well as peripheral bone becomes extremely compact as the antler matures. A sheath of undifferentiated connective tissue, the fibrocellular periosteal layer (II), surrounds the osseous shaft

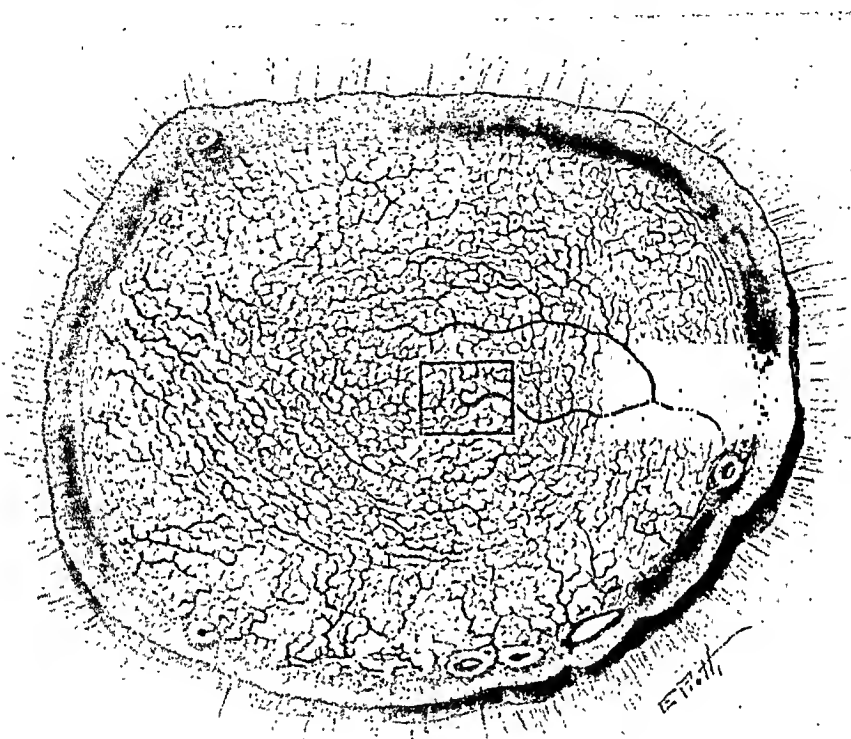


Fig. 2 Drawing of a thick section of antler cleared by the Spalteholz method with the blood vessels injected with India ink, showing a deep recurrent arteriole penetrating the beam of an August antler. The major branches are characteristically wider than the trunk, and communicate freely with the medullary sinuses. (Details of communications within the small rectangle are shown in fig. 3.) In this transverse section, from the distal one-third of the antler beam, reorganization of bone is in its initial stages. $\times 3$.

and becomes continuous with the germinal cap at the tip (I). Rich networks of fine vessels, the *external* and *internal capillary plexuses*, lie on either surface of the periosteal layer except over the growing tip where the internal plexus is lacking.

The periosteum is covered by the skin or "velvet," the innermost layer of which constitutes a vascular lamina containing the large afferent and efferent blood vessels. Ten or 12 arteries, branches of the superficial temporal artery, ascend each antler. They supply the velvet and the bone and branch profusely over the tips to provide a rich blood supply for the areas of rapid growth. These vessels are peculiar (fig. 15) in



Fig. 3 Portion of figure 2 within the rectangular frame enlarged to show the communications between branches of a deep recurrent arteriole (black) and the medullary sinuses (gray). $\times 15$.

that their walls are remarkably thick and are composed of interlacing elastic, muscular and collagenous elements with no distinct elastica interna or externa (Wislocki and Singer, '46). Toward the ends of the antler, the arteries of the vascular lamina of the corium branch and anastomose richly with one another, giving rise to an arterial plexus surrounding the growing tips. Here, relatively large arteries are frequently seen passing over the tip to anastomose with vessels from the opposite surface. A similar number of thin-walled veins accompany the arteries and empty into the superficial temporal

veins. They receive tributaries from both the velvet and the bony substance of the antler. At the antler base, some of the arteries and veins pass through tunnels or grooves between the bony nodules of the corona. Further details regarding the courses of the main arteries and veins and their relationships are presented by Wislocki ('42).

From branches of the main arteries recurrent arterioles are given off which traverse the periosteal layer to penetrate the antler shaft. Along the surface of the entire bony shaft and at the circumference of the growing tip, small *superficial recurrent arterioles* supply the peripheral portions of the bone with blood (zones IV and VI). *Deep recurrent arterioles*, similar in nature but much less numerous, have larger and longer terminal portions which penetrate the central core of the antler where they branch and empty into the thin-walled medullary sinuses (figs. 2 and 3). Both the superficial and deep recurrent arterioles form slender loops consisting of ascending and descending limbs connected by a curved portion which spans the periosteal layer. At the growing tip the ascending and descending limbs are short, but they become progressively longer toward the antler base. At the center of the growing tip, newly formed *capillary retia* are present in great profusion. The retia penetrate the germinal cap (I) in the proximal half of which they become transformed into individual *efferent arterioles*. The latter pass abruptly into the medullary sinuses of the preosseous zone (III).

The *medullary sinuses* constitute an extensive system of thin-walled, anastomosing, longitudinal channels located in the interior of the antler (V). During the initial phase of the sprouting of the antlers some of the sinuses at the base communicate with the venous channels of the bony pedicle (fig. 18).

As the antler increases in length, extensive *venous collecting systems* are formed at intervals, which return the blood directly to the vascular layer of the velvet (fig. 1 and figs. 20 and 21), soon supplanting the initial venous drainage by way of the pedicle. These efferent veins are large and numer-

ous in the distal portion of the antler below the growing tips, but diminish in size and number in the compact bone near the base. In this connection it should be recalled that the bone of the antler, when initially laid down, is cancellous, but that as growth proceeds the older proximal part of the shaft becomes progressively more compact. This increased density involves not only the peripheral zone of the shaft but the entire base of the antler, and is seen to be well advanced as early as mid-June (fig. 19). Progressive ossification appears gradually to encroach upon the avenues of exit of the veins from the interior, resulting in the early disappearance of nearly all vascular connections between the base of the antler and the interior of the pedicle.

In addition to the extensive venous collecting systems, numerous small *superficial venules* drain the peripheral compact bone, passing outward to enter the veins of the velvet. In contrast to the recurrent arterioles, it will be observed that the superficial venules and the trunks of the venous collecting systems cross the periosteal layer in a straight course or with very short loops (fig. 1). Valves have not been observed in the venous channels of the antler.

In addition to the important afferent and efferent channels enumerated above, there are many very minute arterioles and venules present in the Haversian systems of the compact outer part of the bone (*Haversian arterioles and venules*). Moreover, occasional minute arterioles which are not shown in figure 1 are discernible among the sinuses in the deep interior of the antler. At the base of the antler, after the bone has become compact, minute arterioles and venules persist which communicate locally through the pedicle (*external and internal arterioles and venules* of the pedicle).

At the growing tips and on the sides of the antler, the arteries and veins of the velvet give off branches to the external capillary plexus which lies at the junction of the velvet with the outer surface of the periosteal layer. This plexus is well filled with ink in all of our specimens and is recognizable as a narrow, dark zone at the inner surface of the velvet in lon-

gitudinal sections (figs. 1, 8 and 11). When examined in tangential sections, the pattern of the external plexus over the tip of a tine indicates that active formation of new vessels is taking place there (figs. 12 and 13).

As described by Wislocki ('42), and also observed in our specimens whenever the injection was incomplete, the numerous small vessels traversing the germinal cap appear to be simple and unbranched, resembling the recurrent arterioles. However, better injected specimens obtained in the present study have shown these vessels to be much more complex than previously thought. Instead of being single discrete vessels, they are in fact capillary retia (fig. 11, c. r.) associated with, and extensions of, the external capillary plexus. These penetrating retia consist of several more or less parallel channels with many interconnecting links (figs. 12 and 13). Near the middle of the germinal zone, a main channel takes precedence in each rete, while the subsidiary channels regress and disappear. This results in the formation of a single, sparsely branched, efferent arteriole (fig. 11, e. a.) extending down into the proximal half of the germinal cap. These slender efferent arterioles appear to be constricted and, unless the specimens are well injected, not much ink passes into them. In stained sections the lumen is narrow and the walls are seen to be composed of conspicuous endothelial cells surrounded by enlarged stellate cells embedded in a sheath of collagenous fibers (figs. 16 and 17). Some of these satellite cells, or pericytes, are oriented perpendicularly to the axis of the vessel, suggesting that they may be connected with or derived from the vascular endothelium. In the preosseous zone the arterioles pass abruptly into the dilated, thin-walled, medullary sinuses which increase in size and complexity as one proceeds downward.

The pattern of the recurrent arterioles in different regions (fig. 1) appears to depend upon differential rates of growth of the various components of the antler. It has been pointed out that growth of the beam of the antler and of the tines is linear, material being added at the distal extremities, while

the diameter at any level once established does not increase significantly during subsequent growth. Within the germinal cap cellular proliferation is rapid and, since the deeper layers of mesenchymal cells are constantly differentiating into pre-osseous tissue, the germinal cap moves distally as the antler grows in length. Keeping pace with the increase in length, vascular retia are elaborated by the external capillary plexus, transformed into efferent arterioles within the germinal cap, and finally transformed into medullary sinuses in the pre-osseous zone. The pattern of these vessels is further modified by growth of the velvet which overlies the antler tip. With the germinal cap and osseous shaft of the antler pushing upward, carrying the apex farther distally, the velvet grows rapidly, in effect moving away from the center and continuously passing from the circumference of the rounded tip to the vertical sides of the antler shaft. In the course of these changes the external capillary plexus and the origins of the newly sprouted capillary retia appear to follow the velvet. As a consequence, the retia become curved and shift toward the sides of the antler. The retia at the center of the tip are least affected by these changes, whereas, those toward the circumference are gradually transformed into recurrent arterioles (fig. 11).

Further modification of the recurrent arterioles along the sides of the antler (fig. 1) should also be attributed to differential growth of the tissues in which they lie. As a result of calcification of the antler shaft, the terminal portions of these vessels become fixed at the points where they enter the bone. The origins of the recurrent arterioles, which lie in the velvet, likewise tend to be constant since growth of the velvet takes place chiefly at the antler tip. The periosteal layer, on the contrary, appears to grow interstitially throughout its length, although at a slower rate near the antler base than at the tip. As a result, the recurrent arterioles which cross the periosteal layer are drawn out into loops which become steadily longer as one approaches the base of the antler.

In contrast to the recurrent arterioles, the corresponding venules and veins crossing the periosteal layer possess very short loops indicating that they differentiate later than the arterioles, after the process of differential growth in the velvet, periosteum and underlying bone has begun to subside.

Veins or venules are encountered only very occasionally in the periosteal layer and the underlying germinal cap of the growing tips. In figure 17, such an exceptional thin-walled venule is seen in the germinal zone running parallel to a typical efferent arteriole.

Both the efferent arterioles at the antler tip and the *deep* recurrent arterioles at the sides empty into the medullary sinuses in the core of the antler. The *superficial* recurrent arterioles, on the other hand, supply mainly the peripheral or external layer of the bony antler. Distally, in the external portion of the cancellous bone they connect with capillary beds which arise there and are interposed between the superficial recurrent arterioles and the medullary sinuses (fig. 1*). As ossification progresses and the peripheral part of the bony shaft becomes reorganized and more densely calcified, these capillaries lose most of their connections with the medullary sinuses and are drained instead by the superficial venules which cross the periosteal layer to return blood to the veins of the velvet. Along the shaft and at the base of the growing antler the superficial recurrent arterioles and venules connect with the capillary beds of the Haversian canals, thus providing blood supply and drainage for the compact peripheral bone.

As the growing tips near completion (fig. 8), only vestiges of the capillary retia and the efferent arterioles are to be seen. The germinal cap has disappeared, leaving a periosteal layer of uniform thickness continuous with that at the sides of the antler, and an inner capillary plexus has differentiated over the bony tip. The bone has become compact throughout and is vascularized by superficial recurrent arterioles and venules crossing the periosteal layer to communicate with vessels of the Haversian systems (fig. 14). Throughout the bulk

of the completed antler and its tines a core of coarsely cancellous bone remains, containing marrow spaces. This core is now completely surrounded by compact bone, across the antler base as well as at the tips of the tines. As a consequence, the afferent and efferent blood vessels supplying the medullary sinuses have been greatly reduced, and it is probable that the flow of blood in the core of the antler has diminished nearly to the point of stagnation. This belief is strengthened by the observation that in stained, histological sections of antlers obtained in August complete necrosis of the contents of many of the medullary spaces is evident.

Two antler specimens taken during the brief period while the velvet was being shed are noteworthy. One, obtained on September 21 from a wild-shot buck, was injected with India ink through the common carotid arteries. Decalcified sections of this antler showed no gross evidence of ink above the corona except in occasional large vessels in the disintegrating velvet. Stained sections viewed at higher magnification showed ink just above the base of the antler for a distance of 1 to 2 inches in the vessels of the velvet and of the Haversian canals. Some of the ink appeared to have reached these vessels via a few still intact arteries in the partially disintegrated velvet, but some may also have got there by way of minute connections with the external and internal arterioles of the pedicle. A few small channels in the core of the antler base communicated with those of the pedicle but contained no ink.

A second specimen obtained when the velvet was partially shed afforded a study of the surface of the antler. After the velvet had been removed, the surface was rough and lusterless with a multitude of small depressions and elevations apparent to the naked eye (fig. 9). A few small apertures were seen along the beam and at the bases of the tines. Under a low-power dissecting microscope many minute pores containing broken-off blood vessels could be seen opening upon the surface of the antler. The majority of these orifices were inclined distally and the vessels lodged in them were undoubtedly the

small superficial recurrent arterioles and venules previously described. In contrast to antlers which have just shed their velvet, older antlers which have been worn and polished exhibit smooth, shiny surfaces (fig. 10).

In the many specimens of mature antlers which we have examined, there is a conspicuous absence of apertures large enough to transmit the trunks of the numerous venous collecting systems which were present in earlier stages of development. From this observation, it must be concluded that those important avenues for the return of venous blood from the interior of the antler have been almost entirely eliminated by the time calcification has been completed. Their disappearance undoubtedly coincides with the gradual cessation of flow and the stagnation of blood observed in the medullary sinuses during August.

Wislocki ('42) suggested that arteriovenous anastomoses may occur in the vessels of the velvet. The present injected material is not particularly suitable for the investigation of this question, and consequently we have not searched our preparations carefully with this in mind. On the other hand, arteriovenous anastomoses can sometimes be identified in stained histological sections. Weatherford and Wislocki (unpublished observation) have recorded the presence of a blood vessel in the velvet having the histological characteristics of an arteriovenous connection. Furthermore, several other features pertaining to the circulation are compatible with the assumption that arteriovenous anastomoses are present. The first of these is the disparity between the exceptionally large and extensive plexuses of arteries and veins at the antler tips and the limited size and capacity of the newly forming vascular beds of the overlying velvet and underlying germinal cap. The other is the unusual warmth of growing antler tips as appreciated by the hand, an observation remarked upon by numerous observers and one which suggests that the flow of blood through the velvet is both copious and rapid. Arteriovenous anastomoses are known to occur in other parts of the

body where similar conditions of size of vessels and magnitude of flow prevail.

Ligation of growing antlers. Ligatures were placed on several growing antlers to determine the effects of local vascular constriction and, specifically, to see whether shedding of the velvet would result.

On June 13, 1946, two ligatures were placed upon the growing antlers of no. 175, a normal 3-year old male Virginia deer with antlers 4 to 5 inches long. The right ligature was tied just above the first tine, the left, $3/4$ inch above the corona. Both ligatures sank deeply into the velvet but did not cut it.

Ligation was followed by swelling distal to the ties which increased for about a week before it began to subside. While swollen, the antlers were observed to bleed when bitten by flies. Two and one-half weeks after ligation the swelling had subsided and the antlers seemed to be growing normally, though the ligatures were still in place, deeply embedded in the velvet. Six weeks after ligation the antlers were 12 inches long and appeared normal in every respect except for the presence of the embedded ligatures. By the time the animal was killed on August 16, the antlers had attained a length of 14 inches (fig. 4).

The antlers showed no evidence of any alteration in the bone and velvet distal to the ligatures and only minor changes at the sites of ligation. The velvet immediately beneath the ligatures was found to be abnormally thin and hairless, its thickness being but little greater than the diameters of the larger vessels traversing it. Ridges and burr-like exostoses in excess of those normally present on the surface of the antler were evident both proximal and distal to the ligatures. The diameter of the bony shaft of the antler was slightly decreased immediately beneath the ligatures, suggesting that some remodeling of the bone had relieved pressure upon the blood vessels and the velvet. There is no way of knowing exactly when this adjustment occurred, but it seems reasonable to assume that it coincided with the subsidence of swelling. At the time the animal was killed, 9 weeks after ligation,

the arteries seemed to be normal in respect to size and histological structure and the circulation appeared to have been completely restored. This is further attested by the vigorous and essentially normal growth of the antlers. Collateral vascular channels by-passing the constricted regions of the velvet may have arisen temporarily but, if so, no traces of them were evident at the time the animal was killed.

Whereas ligatures were placed on the antlers of no. 175 in June, those of nos. 293 and 291 were ligated late in July. In contrast to the swelling and other features observed in the former, the latter experiments produced little or no swelling, no further growth of the antlers distal to the ligatures, but quite evident necrosis.

On July 24, 1947, the right antler of no. 293 was tied firmly $3/4$ inch above the corona. This yearling deer had at the time a normal first set of antlers 4 to 5 inches long and beginning to branch at the tip. Six days later the ligated antler was amputated. It was found to be only slightly swollen distal to the ligature which was securely in place and deeply embedded in the velvet. A broad band of rough, dry velvet, with a crusty surface and a dark reddish brown appearance had arisen along the posterior surface of the antler from the ligature to the antler tip where it terminated in a cap.

On splitting the antler longitudinally, the velvet included in this band was seen to be redder, thinner and more fibrous than that elsewhere. The periosteal tissues at the tip and sides of the antler distal to the ligature showed focal edema and hemorrhage. Histologic preparations confirmed the gross appearance of necrosis of the velvet. At the site of the constricting ligature the velvet was severely compressed and the blood vessels appeared to be totally occluded. Moreover, there was only slight evidence of beginning resorption of the bone, and no significant reduction in diameter of the bony shaft had occurred.

On July 30, 1947, the left antler of no. 291 was ligated $3/4$ inch above the corona. This animal was a yearling male having a slim first set of antlers about 6 inches long (fig. 5).

Seven weeks after ligation, it was killed and the blood vessels of the antlers were injected with India ink. There had been no swelling and no growth of the ligated portion of the antler during this period, and the velvet had become dark, hairless and shrunken (fig. 6). To the touch it was hard, dry and quite lifeless. The ligatures were still in place though they were loose due to the shrinkage of the tissue beneath them. Upon splitting the decalcified antler with a knife, the velvet distal to the tie was seen to contain dried-up blood, and the decalcified bony core appeared to be more fibrous and woody than normal. No ink was encountered in the blood vessels distal to the ligature.

The results of the ligation experiments may be summarized as follows: ligation in mid-June produced a marked transient swelling of the velvet distal to the tie, remodeling of the bone beneath the tie, and enlargement of the bony rugae just above and below the ligatures, but no permanent interference with either blood supply or growth. Ligation in late July produced exactly opposite results: no swelling, no remodeling of bone, complete interruption of the blood supply distal to the tie with cessation of growth and death of the antler. The dried-up velvet was not shed but remained firmly adherent to the antler.

Bleeding of antlers due to trauma or surgery. On numerous occasions we have observed that bleeding is minimal following injuries to growing antlers. For example, in June of 1947 a three-year old buck with 12-inch antlers became excited and charged a wire fence breaking off about one inch of the tip of the left antler. Although the fracture of the antler was so sudden and severe that pieces of bone were completely evulsed through a split in the velvet, the bleeding which occurred consisted merely of a slow dripping of blood for some minutes.

It has been noted also that very little bleeding is encountered in the course of surgical operations; incising a growing antler tip elicits a slow bleeding but never any spurting of blood.

During an operation (in late July) in which an antler was converted into a tube graft (fig. 5), a tourniquet was placed

just above the left corona before extensive incisions were made in the velvet, the tip of the antler was amputated and the velvet was stripped back from the core of the antler to within $1/2$ inch of the corona. Throughout these procedures bleeding consisted solely of a slow oozing of blood. At this stage the tourniquet was removed while the denuded bony shaft of the antler was resected in three 1-inch sections. After each ablation the oozing of blood from the center of the core increased. Nevertheless, the last cut which left a surface less than $1/2$ inch above the corona showed surprisingly little bleeding, considering the number of blood vessels which must have been transected.

In a second animal a similar resection of the antler was performed without any tourniquet being used. Again the loss of blood was negligible. An interesting comparison was afforded since in this operation the outer table of the frontal bone was removed at a site in the middle of the forehead; here the flow of blood was much faster and more copious than at any stage of operation on the antlers.

The absence of severe bleeding under these various conditions calls for an explanation. Wislocki and Singer ('46) have shown that the principal arteries of the velvet possess relatively small lumens and exceptionally thick walls composed of interweaving muscular and elastic fibers (fig. 15). They have suggested that these structural peculiarities provide for the constriction of the vessels and the reduction of blood flow at the time of shedding of the velvet. The present observations on the relative lack of bleeding following rupture or transection of these vessels substantiates such a concept. It seems probable that their extremely thick, muscular walls constrict instantly and forcibly in response to mechanical stimuli, effectively shutting off the flow of blood.

The blood vessels of the antlers, more especially at the growing tips, are relatively large and numerous and consequently the flow through them must normally be relatively great. As pointed out in a preceding passage, the unusual warmth of the growing antlers also probably indicates that a

relatively large amount of blood passes through them. Add to this the fact that the vessels are located in the velvet where they are particularly susceptible to traumatic injury and it will at once become apparent that a mechanism for their prompt and effective closure is of considerable importance.

DISCUSSION

Changes in the blood supply of the antler during growth. From the material at hand, differences of opinion regarding blood supply of the antler are understandable since (1) the many connections between the vessels of the velvet and the interior of the antler are not seen unless the blood vessels are completely injected, and (2) both the blood supply and drainage change as the antler grows and matures. Macewen ('20), Noback ('29), Noback and Modell ('30), and Modell and Noback ('31) concluded that blood reached the growing antler primarily through internal channels derived from the pedicle and the frontal bone. Caton (1877) gave about equal value to an internal supply and to external sources derived from the velvet and from the skin over the pedicle. Wislocki ('42) concluded that the antler was vascularized almost exclusively by vessels supplying it externally through the velvet and that internal channels were of negligible importance.

Our findings show that the rapidly growing antler tips are supplied primarily by arteries from the overlying velvet during all stages of growth. In the early stages (April, May and June), direct arterial communications also exist between the antler and the pedicle, but these diminish rapidly as the bone across the antler base increases in density. Both the swelling of the velvet which followed ligation of the antler in mid-June and the continued viability of the antler attest the presence of an internal supply at that time. Sectioned material, however, shows that by mid-June the channels for such supply have already become much diminished. By late July the external vessels have become the only ones of significance, and their closure by ligation resulted in death of the

antler, the internal supply at that stage being barely sufficient to produce a slight swelling immediately after ligation.

Venous drainage likewise becomes modified as the antlers grow, but here the changes are even greater than in the case of the arteries since in the very early stages of growth drainage takes place almost entirely through internal channels. With the advent of the venous collecting systems, the blood is returned from the antler core directly to the velvet, these avenues becoming of increasing importance as calcification closes the efferent channels through the antler base. By mid-July, the bulk of efferent flow is external, through the vessels of the velvet.

Our findings emphasize the importance of the multitude of small vessels connecting the arteries of the velvet with the substance of the antler at all stages of growth and are in essential agreement with the findings of Rhumbler ('29), and Wislocki ('42) who described these vessels in injected specimens. Caton (1877) in his shrewd analysis of gross, un-injected material also recognized their existence and rightly judged them to be important, although Macewen ('20), probably because of inadequate injection techniques, specifically denied their existence. We have pointed out, in addition, that newly formed capillary retia are present at the center of the antler tip. These, together with the efferent arterioles which arise from them, are not only the source of the recurrent arterioles and medullary sinuses, but are at the same time important channels carrying blood from the velvet to the antler core. Modell and Noback ('31) also described newly formed vessels in the growing tip, but judged them to be occluded at their origins and otherwise filled with blood derived from the interior of the pedicle.

Our interpretation of the continuous vasenlar transformations concomitant with growth at the antler tip is as follows. At the center of the tip (1) capillary retia are elaborated from the indifferent external capillary plexus overlying the germinal cap. (2) in the interior of the germinal cap the retia are transformed into efferent arterioles, and (3) in the

preosseous zone the efferent arterioles are, in turn, transformed into medullary sinuses destined to occupy the core of the mature bony antler. At the periphery of the growing tip the retia follow a similar course but, unlike those at the center, they become curved near their origins and subsequently pass to the sides of the antler as typical recurrent arterioles. Here, they supply blood to the peripheral bone that is destined to become the compact, outer shell of the antler as it matures.

This sequence of growth and differentiation of vascular elements within the antler tip is not only continuous but proceeds with a rapidity that is possibly unique among animal tissues. Wislocki and Singer ('46) have discussed the speed of antler growth, estimating the daily increase in length of elk and caribou antlers to be as great as 1.5 to 2.0 cm. Virginia deer, having smaller antlers, do not equal this record but several animals under observation have shown daily increments of 4.0 to 6.0 mm, an amount roughly equal to the thickness of the germinal cap. Consequently, the new growth of capillary retia and the modifications which transform them into typical recurrent arterioles or medullary sinuses must, of necessity, occur with extreme rapidity to keep pace with the elongation of the antler.

Modell and Noback ('31) and Wislocki, Weatherford and Singer ('47) have given histological descriptions of the walls of the arterioles and the surrounding mesenchyme within the germinal cap with which our findings are in agreement. Modell and Noback ('31), however, placed particular emphasis upon the extra-endothelial cells lying along the walls of the arterioles and oriented perpendicularly to them. They took these to be cells migrating from the vessel walls and concluded that they were the source of mesenchyme of the germinal cap. Both interpretation and conclusion are placed in doubt when it is considered that the newly formed retia in the outer half of the germinal cap become transformed to simple arterioles in the inner half. As shown by Clark and Clark ('40), the transformation of similar retia into arterioles in the

rabbit's ear takes place through the increasing dominance of one or more of the capillary channels and the accompanying elimination of the most of the subsidiary channels. As the subsidiary channels disappear, adventitial cells from their walls are often left behind in the tissue, and these frequently are to be seen lying at right angles to the walls of the remaining channels. In the light of the Clarks' observations, it seems preferable to conclude that the pericytes or extra-endothelial cells surrounding the arterioles of the germinal cap are the natural result of circulatory modifications involving the reduction and elimination of a capillary net.

Role of the circulation in shedding of the velvet. Most writers on the subject have agreed that circulatory changes in some way underlie disintegration and shedding of the velvet but have disagreed as to the mechanisms involved. Caton (1877), Wislocki ('42) and others have effectively discredited the widely held view espoused by Macewen ('20) that bony accretions at the corona cause compression and local occlusion of the major vessels and the consequent death and shedding of the velvet. The results of our ligation experiments are also incompatible with this view. Caton (1877) believed the velvet to be shed when the blood supply to the growing antler was cut off by solidification of the completed antler, beginning at the tips and progressing down the branches and the beam until "the passages through the surface of the antler, which admitted the circulation from the periosteum, have become closed, soon after which the velvet is discarded." Caton also suggested that the vessels supplying the velvet might close of themselves, not by mechanical compression at the burr but in obedience to some law of nature not clearly understood. Wislocki ('43) suggested that shedding of the velvet was related to gonadal activity, specifically to the testosterone level in normal males. Subsequent experiments (Wislocki, Aub and Waldo, '47) have demonstrated that shedding of the velvet can be induced by the administration of testosterone or estradiol to either normal or castrate males at any time while the antlers are growing.

Completion of growth at the antler tips may normally play an important role in initiating shedding of the velvet, but it has been amply demonstrated in several instances that shedding of the velvet is not necessarily dependent upon this factor. Antlers in normal males given testosterone have been artificially retained for two to 6 months after normal shedding time, the new antlers succeeding them having a correspondingly late start (Wislocki, Aub and Waldo, '47). Yet, in each case the velvet of the new antlers was shed at the usual time in mid-September, although the antler tips were still blunt and consisted of soft, crumbly bone. With the exception of the tips, the antlers were otherwise normally hard and compact.

The bone on the surface of the antler shaft is always compact at the time the velvet is shed, whether the shedding be normal or induced by the administration of steroid hormones. Indeed, bare antlers, resulting from the experimental administration of testosterone or estradiol in early June, have consistently shown a peripheral layer of compact bone 1 to 2 mm thick with a very finely porous surface, in contrast to the open cancellous surface of the normal growing antler at this period. Castrate antlers, normally porous, show a similar compact surface after shedding of the velvet has been hormonally induced. In no case after shedding of the velvet has it been possible to find a significant number of apertures of sufficient size to transmit vessels other than the small arterioles or venules. The large venous collecting systems always become occluded as peripheral calcification increases, effectively interrupting the circulation of blood through the major portion of the substance of the antler. Furthermore, it is possible that the venous stasis in the core of the antler might induce constriction of the thick-walled afferent arteries of the velvet, thereby producing an ischemia of the entire antler. Such an effect would be in keeping with our observations that these vessels are extremely sensitive to mechanical stimuli and respond by constricting. A gradually increasing interference with the circulation appears to lead to softening,

disintegration and shedding of the velvet, whereas the sudden local occlusion caused by ligation is followed by desiccation, hardening and adherence.

SUMMARY

Deer antlers possess a blood supply which changes during growth. Each antler is supplied by 10 to 12 external arteries which arise from the superficial temporal arteries and ascend the antler in the vascular layer of the velvet. During all stages of growth these supply the velvet and the bone with blood and branch profusely over the growing antler tips. Numerous superficial recurrent and deep recurrent arterioles arise from the arteries of the velvet along the entire bony shaft. The former supply the peripheral portions of the bone while the latter, which are longer and larger, penetrate the central core of the antler where they branch and empty into numerous thin-walled medullary sinuses. These recurrent arterioles develop from capillary retia at the growing tips and form slender loops consisting of ascending and descending limbs which become progressively longer toward the antler base.

At the antler tips a multitude of capillary retia arise from an indifferent capillary plexus overlying the germinal cap. These capillary retia penetrate the germinal cap in the proximal half of which they become transformed into individual efferent arterioles. The latter extend down into the preosseous zone where they communicate with the medullary sinuses. The capillary retia thus interposed between the indifferent capillary plexus and the efferent arterioles at the growing tip represent sites at which the growth of new blood vessels occurs.

The medullary sinuses constitute an extensive system of thin-walled longitudinal channels located in the interior of the antler.

When the antler first begins to sprout, venous drainage takes place internally by way of veins of the antler pedicle.

As a result of ossification of the antler base, the internal venous channels diminish to insignificance by the middle of the growing season (July) and are supplanted by venous collecting systems which empty directly into veins of the velvet. Thus, the venous drainage of the antler like the arterial supply occurs principally by way of the blood vessels of the velvet.

As growth of the antler nears completion and the peripheral bone becomes more compact, the trunks of the venous collecting systems become greatly reduced and the blood flow through the antler core becomes correspondingly diminished. Furthermore, as the growing tips approach completion, only vestiges of the capillary retia and the efferent arterioles remain.

Ligatures tied near the antler bases did not produce shedding of the velvet. Ligation in mid-June produced no permanent interference with either blood supply or growth, whereas ligation in July caused cessation of growth and death of the antler. These results indicate that in June there are enough internal vascular channels to by-pass the blood around the site of the ligature, whereas by July such internal channels are no longer available or adequate.

Natural shedding of the velvet appears to be associated with ossification of the peripheral zone of the antler shaft, a process which restricts the venous return from the interior of the antler.

LITERATURE CITED

- CATON, J. D. 1877 *The Antelope and Deer of America*. Hurd and Houghton, Cambridge.
- CLARK, E. R., AND E. L. CLARK 1940 Microscopic observations on the extra-endothelial cells of living mammalian blood vessels. *Am. J. Anat.*, 66: 1-49.
- MACEWEN, WILLIAM 1920 *The Growth and Shedding of the Antler of the Deer*. Maclehose, Jackson and Co., Glasgow. 109 pp.
- MODELL, WALTER, AND C. V. NOBACK 1931 Histogenesis of bone in the growing antler of the Cervidae. *Am. J. Anat.*, 49: 65-86.
- NOBACK, C. V. 1929 The internal structure and seasonal growth changes of deer antlers. *Bull. N. Y. Zool. Soc.*, 32: 34-40.

- NORACK, C. V., AND W. MODELL 1930 Direct bone formation in the antler tines of two of the American Cervidae, Virginia deer (*Odocoileus virginianus*) and Wapiti (*Cervus canadensis*). With an introduction on the gross structure of antlers. *Zoologica*, 11: 19-60.
- RHUMBLE, LUDWIG 1929 Zur Entwicklungsmechanik von Korkziehergeweihbildungen und verwandter Erscheinungen. *Arch. f. Entw.-Mech.*, 119: 441-515.
- WISLOCKI, G. B. 1942 Studies on the growth of deer antlers. I. On the structure and histogenesis of the antlers of the Virginia deer (*Odocoileus virginianus borealis*). *Am. J. Anat.*, 17: 371-415.
- 1943 Studies on growth of deer antlers. II. Seasonal changes in the male reproductive tract of the Virginia deer (*Odocoileus virginianus borealis*) with a discussion of the factors controlling the antler-gonad periodicity. *Essays in Biol.*, in honor of Herbert M. Evans. Univ. Cal. Press, pp. 631-653.
- WISLOCKI, G. B., AND M. SINGER 1946 The occurrence and function of nerves in the growing antlers of deer. *J. Comp. Neurol.*, 85: 1-19.
- WISLOCKI, G. B., J. C. AUB AND C. M. WALDO 1947 The effects of gonadectomy and the administration of testosterone propionate on the growth of antlers in male and female deer. *Endocrinology*, 40: 202-224.
- WISLOCKI, G. B., H. L. WEATHERFORD AND M. SINGER 1947 Osteogenesis of antlers investigated by histological and histochemical methods. *Anat. Rec.*, 99: 265-296.

PLATES

PLATE 1

EXPLANATION OF FIGURES

4 A three-year old male deer (no. 175), showing growth attained by antlers on August 16. Ligatures (indicated by arrows) were placed 9 weeks before, on June 13, when the antlers were 5 inches long. The ligature on the left antler, indistinctly seen in the photograph, lies deeply embedded in the velvet just above the corona.

5 and 6 Deer (no. 291) showing the left antler at the time of ligation, July 30, and 7 weeks later on September 19. Note that the antler failed to grow and that the velvet became thin and shrunken. (The right antler which gives the impression of having been amputated had been converted into a tube graft displacing the growing tip toward the center of the forehead.)

7 Section through the growing tip of a June antler. Fixed in Zenker's fluid and stained with azan. In addition to the velvet (V), the extent of the germinal (G), preosseous (P), and osseous (O) zones is indicated. Note that the fibrocellular periosteal layer (P') merges with the germinal zone over the tip. $\times 2.2$.

8 Section about 1 mm thick through the tip of a nearly completed tine from an August antler (no. 175). The blood vessels were injected with India ink and the antler was decalcified and cleared by the Spalteholz method. Between the velvet and the bone the fibrocellular periosteal layer may be seen forming a light zone of uniform thickness. This layer is bounded internally and externally by narrow, dark zones consisting of the injected internal and external capillary plexuses. Note that the fibrocellular periosteal layer is continuous over the tip and that the germinal, preosseous and cancellous osseous zones of previous stages are absent, having been replaced by compact bone. The three horizontal lines indicate the levels of the sections shown in figures 12 to 14. $\times 4$.

9 Surface of an antler beam (about midway between the base and the tip) from which the velvet has been newly shed. Note the rough, pitted surface. $\times 2.5$.

10 An area of a shed antler, comparable to that illustrated in figure 9, showing the smooth, shiny surface of the antler after it has been polished by continued wear. $\times 2.5$.

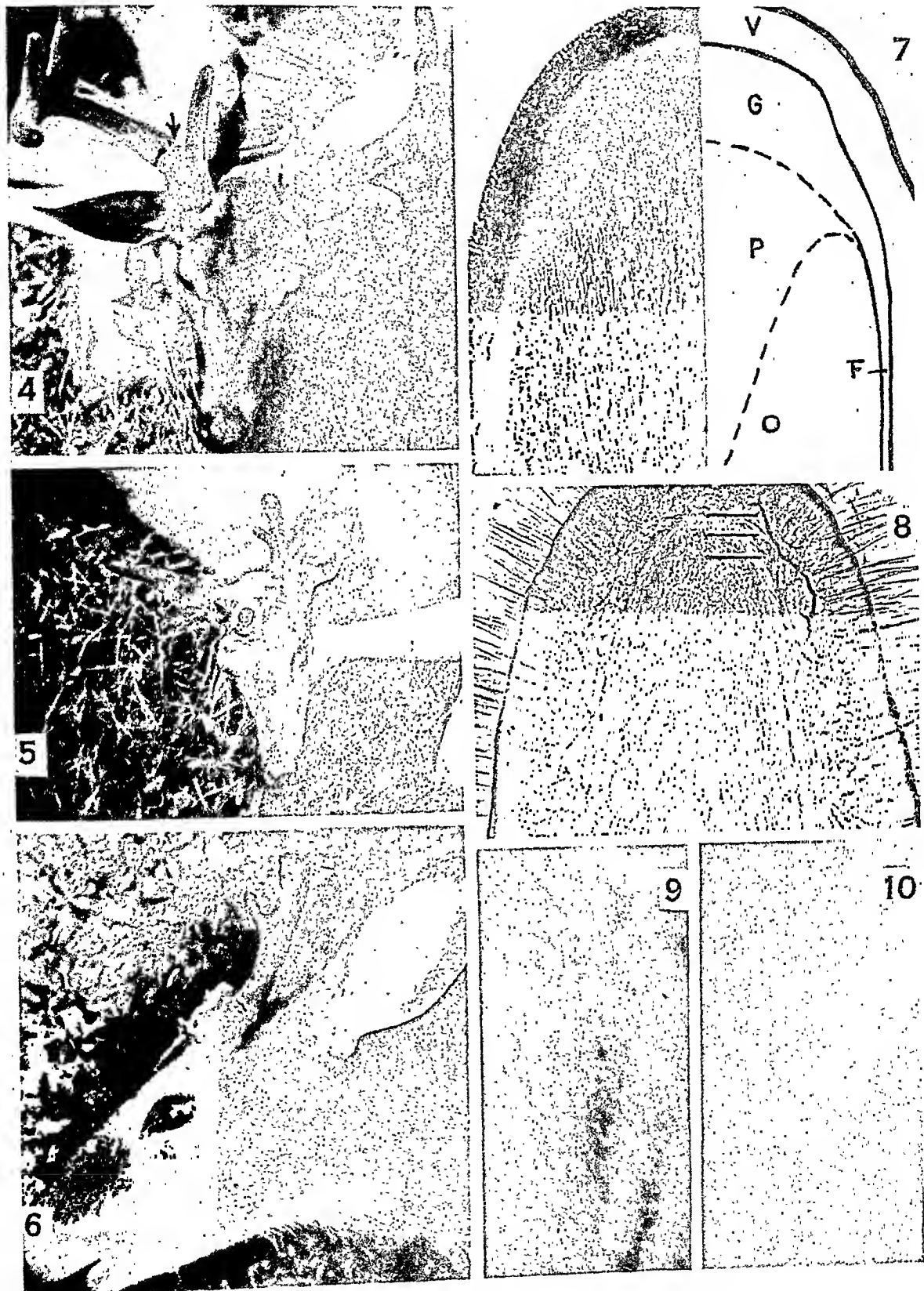


PLATE 2

EXPLANATION OF FIGURES

11. A section, about 1 mm thick, through the actively growing tip of an August antler (no. 175). The blood vessels were injected with India ink, and the antler was decalcified, sectioned, and cleared by the Spalteholz method. The different zones can be visualized by reference to figure 7. The capillary retina (c.r.) are visible in the upper and darker half of the germinal zone. Note that toward the sides of the tip, particularly on the right side of the figure, the retina has become greatly simplified and the persisting vessels have assumed the characteristic shape of recurrent arterioles. The efferent arterioles (e.a.) which arise from the retina and occupy the lower half of the germinal cap are constricted and consequently contain little ink. Their courses may be followed indistinctly to their junctions with the very dark medullary sinuses (m.s.) lying directly below the germinal cap. Toward the bottom of the figure peripheral bone is present on either side of the dark core of medullary sinuses. Here the superficial recurrent arterioles communicate with capillaries which in turn empty into the medullary sinuses (fig. 1*). Few venous channels of any sort exist in the germinal cap or cross the periosteal layer at this level. Sections of several large arteries may be seen in the deep layer of the velvet on the left side of the figure and directly over the germinal cap. A section of a large vein is visible in the velvet on the right side of the figure. Within the germinal cap, the retina and the efferent arterioles bulge away from the center indicating lateral growth within the cap. In the lower left-hand corner of the figure, medullary sinuses may be seen fanning out into the peripheral bone where they are destined to become greatly modified as the peripheral bone matures and becomes calcified. The medullary sinuses at the center of the core, on the contrary, maintain their longitudinal positions and their identities as the antler increases in length. The rectangular frame in the germinal zone indicates the approximate location of the section shown in figure 16. $\times 10$.

11

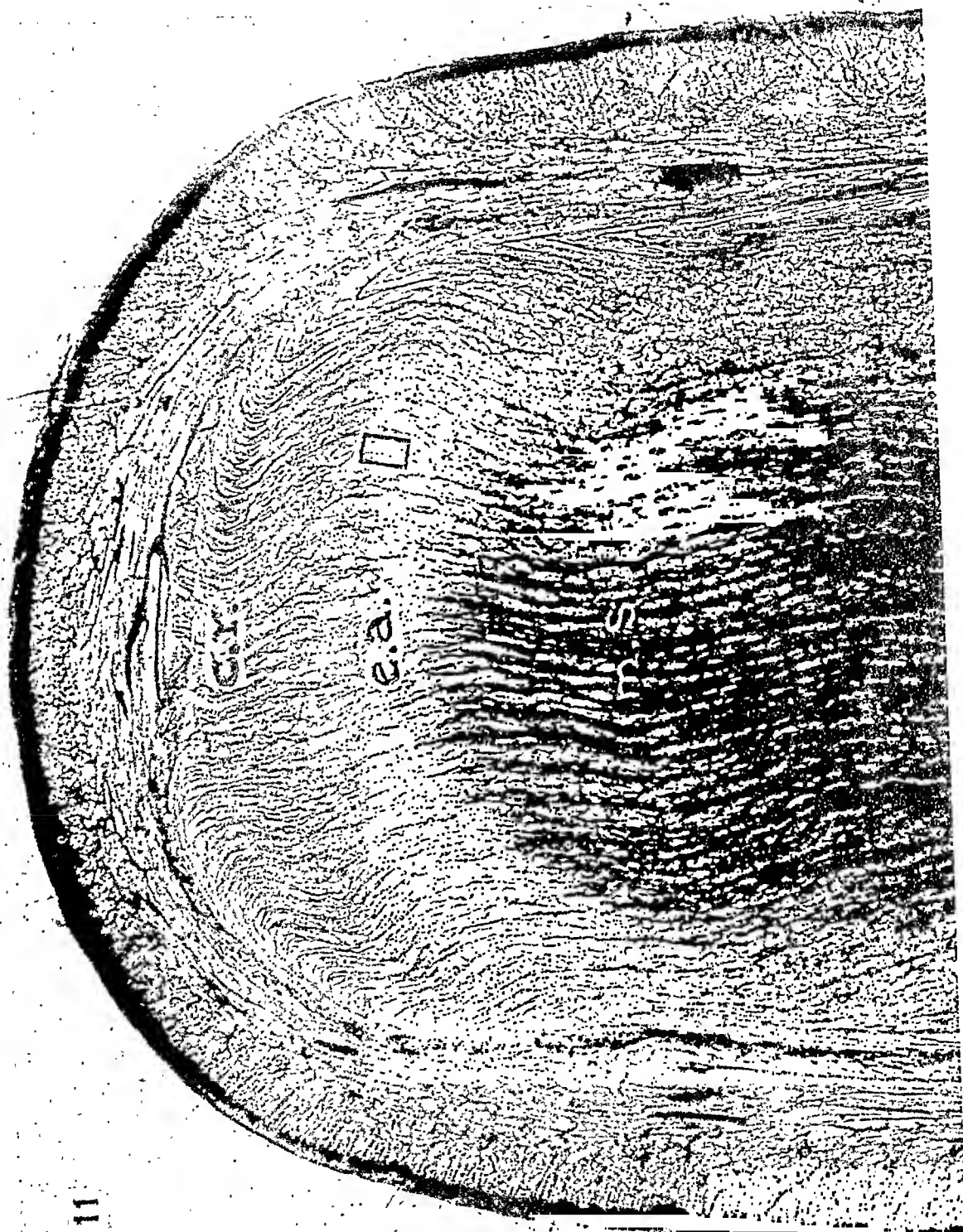


PLATE 3

EXPLANATION OF FIGURES

12 to 14 are transverse sections from the tip of a nearly completed August tine cut at the levels indicated by the three horizontal lines in figure 8. The blood vessels were injected with India ink and the decalcified tine was cut into 200μ sections which were cleared by the Spalteholz method.

12 passes through the external capillary plexus (of the periosteum) which one sees as a corona composed of complex networks of newly formed capillaries (c). In the center are the slightly curved initial portions of the capillary retia (c.r.). In a younger and more actively growing tip as illustrated in figure 11 the proliferating blood vessels are more numerous, although the pattern is the same in principle. $\times 12$.

13 passes through the internal capillary plexus just over the bony tip. Newly formed capillary networks are infrequent at this level and the capillary retia are outnumbered by the single descending limbs of the recurrent arterioles. $\times 12$.

14 passes through the bony tip about 2 mm below its apex. At the periphery of the photograph, the external capillary plexus is visible and medial to it are retia, many of which have been reduced and organized into individual recurrent arterioles. In the center, minute arterioles and capillaries are visible in the irregular marrow spaces of the newly formed compact bone. $\times 12$.

15 A branch of one of the large arteries of the velvet from a June antler showing its typical thick wall. Stained by Pap's silver method for reticulum. The wall of the vessel is composed of interlacing elastic, muscular and collagenous elements with no distinct elastica interna or externa. $\times 160$.

16 Section of an efferent arteriole from the proximal half of the germinal cap of a growing antler illustrative of the region shown in the rectangle in figure 11. Fixed in Zenker's fluid and stained with eosin and methylene blue. The endothelial cells are hypertrophied and are surrounded by enlarged stellate and fusiform pericytes embedded in a sheath of collagenous fibers. Surrounding these is the mesenchyme of the germinal cap. In the upper portion of the figure are satellite cells (X) lying nearly perpendicular to the long axis of the vessel. $\times 240$.

17 Another section from the germinal cap. Specimen impregnated by Bodian's protargol method following fixation in cold acetone, bringing out particularly the shapes of the cells. At the right is seen a constricted efferent arteriole, with hypertrophied cells and numerous pericytes. Lying parallel to it at the left of the figure is a vessel of similar size but possessing thin, delicate walls; this is thought to be a venule. $\times 240$.

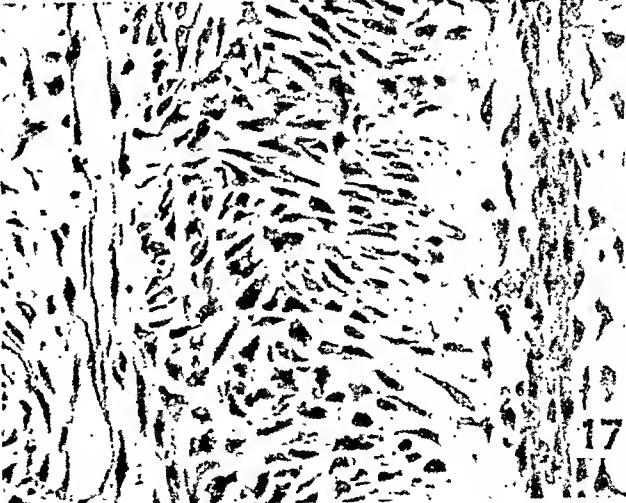
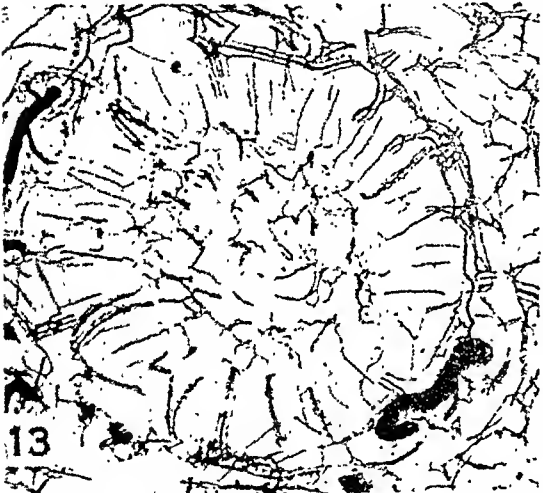
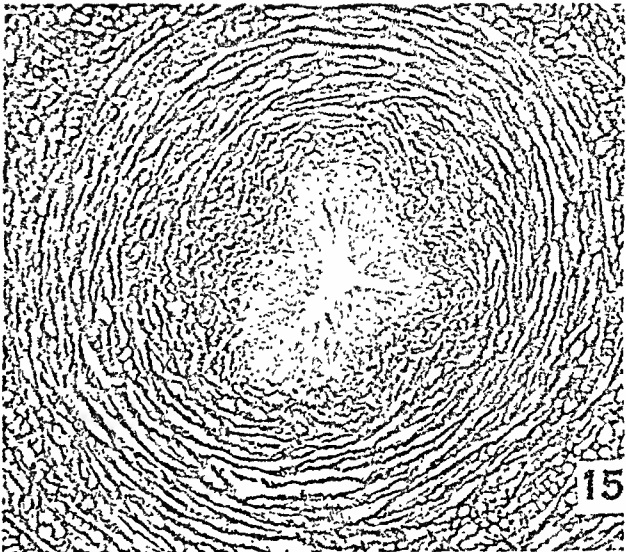
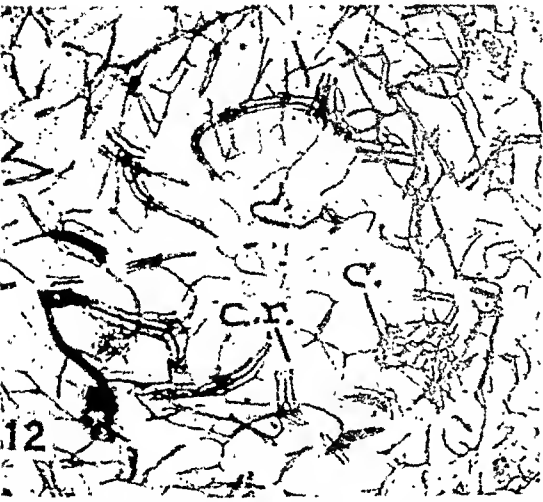


PLATE 4

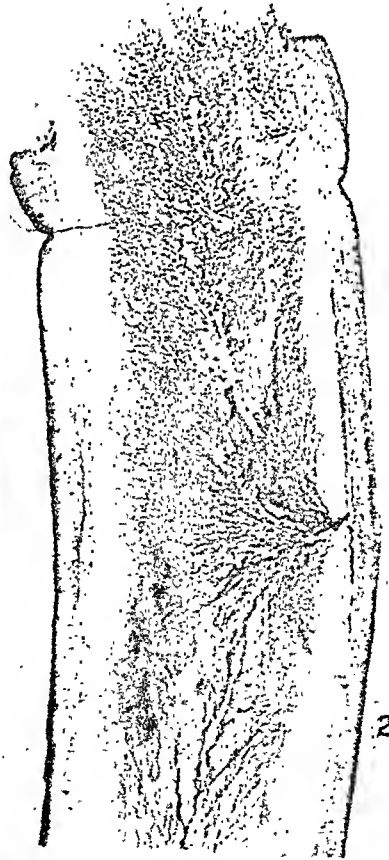
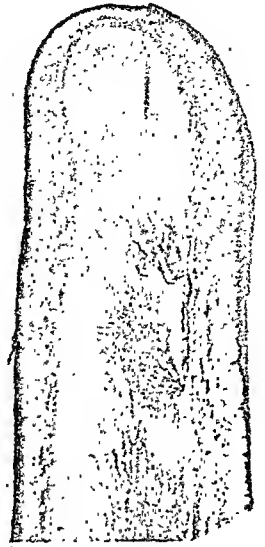
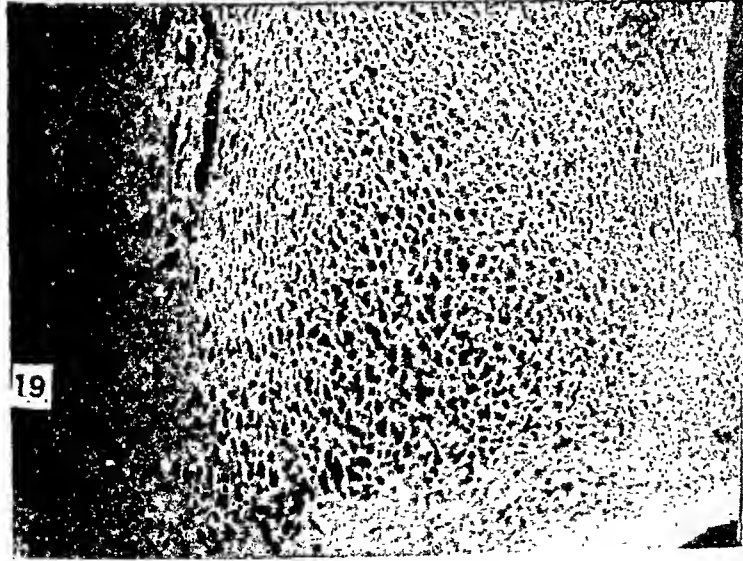
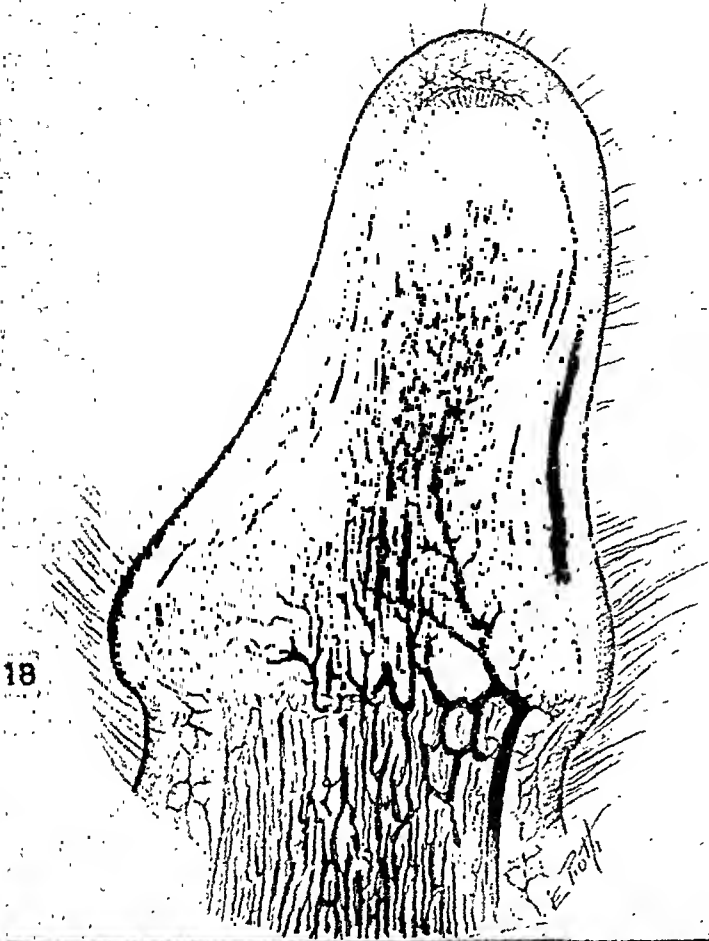
EXPLANATION OF FIGURES

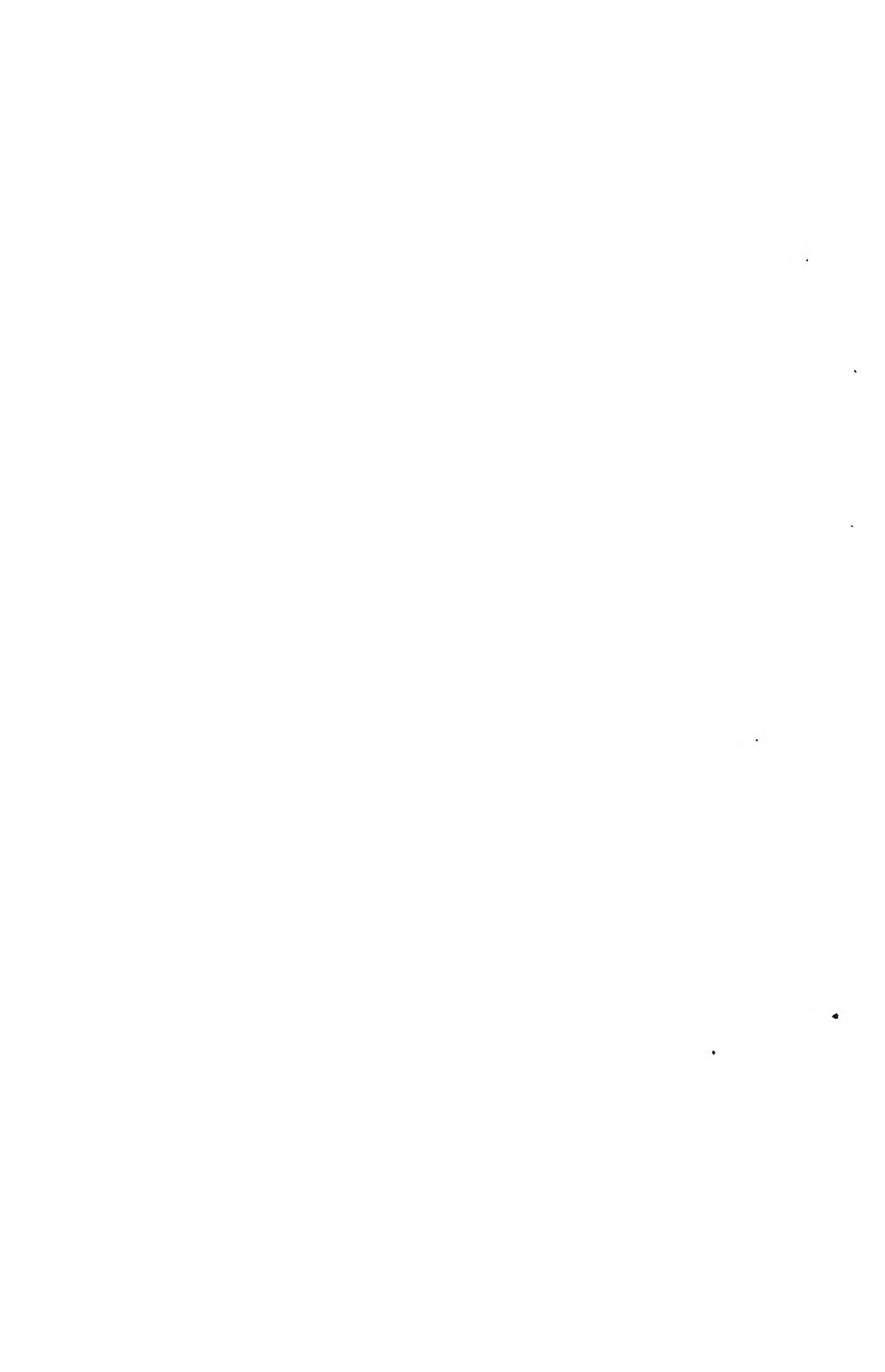
18 Drawing from a mid-longitudinal section of a newly sprouting June antler (Sika deer). Specimen injected with India ink, decalcified, and cleared by the Spalteholz method. The antlers of Sika deer start growing about one month later than those of Virginia deer; hence the stage of development shown would correspond to a May antler for the Virginia deer. Many of the medullary sinuses communicate with venous channels in the pedicle but others empty directly into subcutaneous veins (one of which is visible on the right). The channels seen converging towards this vein represent a venous collecting system in process of formation. $\times 1.5$.

19 Perpendicular section through the base of a mid-June antler which had been macerated, leaving only the bone. The bone at the periphery and that across the base (bottom of photograph) is already much denser than that in the core of the antler. The large spaces in the bone at the left of the figure represent the channels occupied by a venous collecting system, the trunk of which emerged at the surface of the antler just above the corona. $\times 2$.

20 Mid-longitudinal section of a growing tine from a June antler (no. 169). Specimen injected with India ink, decalcified, and cleared by the Spalteholz method. Parts of three venous collecting systems are visible communicating with veins in the inner, vascular layer of the velvet. $\times 1.5$.

21 Section from the beam of the same antler as that shown in figure 20. A large venous collecting system is shown arising from a multitude of medullary sinuses and anastomosing with a contiguous venous collecting system, a part of which appears in the lower portion of the photograph. The tip of this antler was removed and a ligature placed just below the cut end before the injection was made. Consequently, the ink which is present in the medullary sinuses and the collecting systems is presumed to have reached them through the superficial and deep recurrent arterioles. $\times 1.5$.





CYTOCHEMICAL OBSERVATIONS ON THE FETAL MEMBRANES AND PLACENTA OF THE BAT, MYOTIS LUCIFUGUS LUCIFUGUS

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THIRTY-EIGHT FIGURES

This paper completes the account of the author's histochemical survey of the placenta of the bat. Observations on the distribution and significance of cytoplasmic ribose nucleoproteins, glycogen, alkaline, neutral and acid phosphatases, lipase, Bodian protargol reaction, iron, metachromasia and proteinous inclusions of the visceral layer of the yolk sac are presented. An earlier communication describes the distribution and significance of the placental lipids, mitochondria and Golgi apparatus (Wimsatt, '48).

MATERIAL AND METHODS

Fifty-three bats carrying conceptuses ranging in development from implanting blastocysts to fetuses at term were used.

For the study of nucleoproteins 5- μ sections of material fixed in Zenker's or Zenker-formal (Maximow) solutions were stained in eosin and methylene blue. Control sections were incubated before staining in a buffered solution of ribonuclease prepared from beef pancreas according to the method described by Kunitz ('40).

Five- μ sections of material fixed in an absolute alcohol. picric acid and formalin mixture were stained for glycogen by the Bauer-Feulgen method and by the silver impregnation

technique of Mitchell and Wislocki ('44). The specificity of the reactions was checked by means of saliva-digested control slides and by the staining of liver sections.

For the demonstration of iron, 5- μ sections of material fixed in a solution of absolute alcohol and formalin (Seott, '33) were stained by the Turnbull blue reaction (Lison, '36), the Prussian blue reaction, and the Macallum reaction for "organic" iron (Bensley and Bensley, '38).

Phosphatase activity was studied at 3 pH levels (9.4, 7 and 4.7) in material fixed in cold 80% alcohol (alkaline range) and cold acetone (neutral and acid ranges). The pH was adjusted by means of a glass electrode. Five substrates in appropriate veronal or acetate buffers were used: sodium- β -glycerophosphate, ribose nucleic acid (from yeast), the calcium salt of fructose 1-6 diphosphate, adenylic acid and lecithin.¹ For alkaline phosphatase the method of Gomori ('41) was utilized; for neutral and acid phosphatases, that of Wolf, Kabat and Newman ('43).

Lipase was investigated by means of Wachstein's ('45) modification of the method of Gomori ('45) in tissues fixed in cold acetone and sectioned at 5 μ . Control sections of pancreas and liver, tissues known to contain lipase, were always run with the sections of the placenta.

In addition to the silver impregnation method of Mitchell and Wislocki ('44), the Dawson and Barnett ('44) modification of the Bodian protargol method was utilized for the detection of argyrophilic substances. The staining procedure was applied to 5- μ sections of materials fixed in a mixture of 80% alcohol, formalin and acetic acid (Bodian's fixative no. 2); an absolute alcohol, picric acid and formalin mixture; and Bouin's fluid.

For the study of metachromasia 5- μ sections of tissues fixed in an absolute alcohol, picric acid and formalin mixture,

¹ The Na- β -glycerophosphate was obtained from The Will Corporation, Rochester, N. Y., the nucleic acid, adenylic acid and fructose 1-6 diphosphate from the Schwarz Laboratories, Inc., New York 17, N. Y., and the lecithin from the Pfanstiel Chemical Co., Waukegan, Illinois.

Zenker's, and Maximow's fluids were stained in 0.2% toluidine blue by a method devised by B. Sonnenblick (personal communication). Other sections were stained in 1/800 aqueous toluidine blue and mounted in glycerine or glycerine jelly.

Chromophilic globules in the mesothelium of the yolk sac were studied in material fixed in 10% formalin, Zenker's Zenker-formalin, Bouin's and Regaud's fluids, and stained by the Masson triacid mixture, the Azan technique, eosin and methylene blue, hematoxylin and eosin, Mallory's phosphotungstic acid hematoxylin, or Baker's ('46) pyridine extraction test.

OBSERVATIONS

The topography and developmental histology of the fetal membranes and placenta are presented in detail in two earlier papers (Wimsatt, '44 and '45), and summarized in a more recent one (Wimsatt, '48). A text figure from the last paper is here reproduced (fig. 1) for purposes of orientation; it illustrates the definitive arrangement of the placental constituents and also the terminology to be used.

The observations will be presented under the headings of the histochemical methods employed, and will be followed by a discussion and evaluation of the results.

The distribution of cytoplasmic basophilia

In all instances to be described cytoplasmic basophilia was abolished when sections were treated with ribonuclease before being stained in methylene blue. The distribution of cytoplasmic basophilia is therefore presumed to correspond with that of ribose nucleoprotein.

Endometrium. A varying degree of cytoplasmic basophilia is displayed during pregnancy by all of the cellular elements of the endometrium — epithelial cells, connective tissue cells, and endothelium. It is most pronounced in the lining epithelium of the uterus during progestation, where the cytoplasm of the columnar epithelial cells is tinted a uniform deep blue, but with the faint suggestion of a superimposed delicate longi-

tudinal striation in the cytoplasm beneath the nucleus. The moderate mucoid secretion of the surface cells has a very pale basophilic tint, whereas the less copious secretion of the glands is distinctly acidophilic. The glandular cells during progestation are lower than the surface cells and their nuclei are more basally situated. Their cytoplasmic basophilia, while distinc-

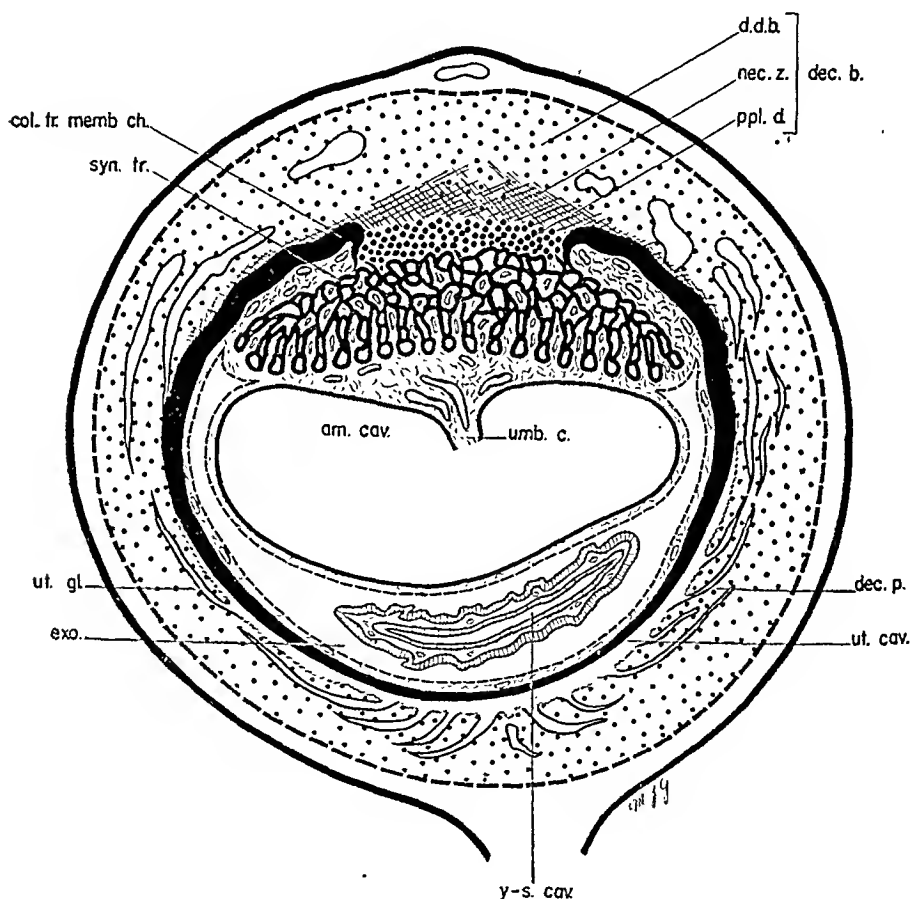


Fig. 1 Semidiagrammatic cross section of the pregnant uterus of the bat showing the definitive arrangement of the discoidal placenta and the fetal membranes.

am. cav., amniotic cavity; col. tr. memb. ch., columnar trophoblast of membranous chorion; d.d.b., deeper zone of decidua basalis; dec. b., decidua basalis; dec. p., decidua parietalis; exo., exocoelom; nec. z., necrotic zone; ppl. d., paraplacental decidua; syn. tr., syncytial trophoblast; umb. c., umbilical cord; ut. c., uterine cavity; ut. gl., uterine gland; y-s. c., yolk-sac cavity.

tive, is perhaps only a little more than half as intense as that of the surface epithelium. A basophilic wash occurs throughout the cytoplasm, but variable numbers of darker blue, irregularly net-like bodies are also present.

Beginning at implantation, the lining epithelium of the uterus is eroded in the vicinity of the ovum, and with the rapid expansion of the chorionic vesicle, is ultimately destroyed completely. Late in pregnancy this epithelium is replaced in the region in apposition with the mesometrial hemisphere of the chorionic vesicle, where the erosive activity of the chorionic trophoblast gradually subsides. The reconstituted epithelium is composed of cuboidal cells whose cytoplasm is characterized in the region of the nucleus by a basophilic wash of moderate intensity (less than in any of the epithelial elements during progestation). The apical cytoplasm stains very faintly or not at all.

The glands persist longer. They disappear fairly early from the decidua basalis antimesometrially, and somewhat later from the lateral walls of the uterus adjoining the equatorial zone of the chorionic vesicle, where the gland ducts nearest the myometrium are the last to disappear. Near mid-gestation the degenerating glandular cells of the antimesometrial zone display a cytoplasmic basophilia of moderate intensity, less than at earlier stages, and less than in the glands of the lateral wall of the uterus at the same stage. The basophilic material is distributed throughout the cytoplasm in the form of irregular granules and ill-defined net-like accumulations. The more intense basophilia of the glandular cells in the lateral and mesometrial walls of the uterus tends to be most concentrated perinuclearly in the basal cytoplasm. As the degeneration of the glandular cells continues, their cytoplasmic basophilia gradually diminishes and usually disappears before the cells completely disintegrate.

With the exception of the progestational secretion of the lining epithelium of the uterus already mentioned, basophilia in the uterine and glandular lumens at all stages is restricted to the nuclear fragments of epithelial cells and leucocytes, and

is unaffected by ribonuclease. The remaining detritus, which in earlier stages is often copious in amount, is intensely acidophilic.

The connective tissue cells of the decidua differ in the degree of hypertrophy which they undergo and in their histochemical reactions. In the paraplacental layer of the basalis (ppl. d. in fig. 1), the cells become maximally hypertrophied and vacuolated; in the deeper zone of the basalis, extending from the paraplacental layer to the myometrium (d.d.b. in fig. 1), the cells become hypertrophied, but remain relatively non-vacuolated; in the decidua parietalis (dec. p. in fig. 1) the cells undergo the least hypertrophy. The cells of these three regions differ in their content of cytoplasmic basophilic material. The swollen cells of the paraplacental layer display the least degree of cytoplasmic basophilia; those of the deeper decidua basalis, a conspicuously greater degree, but the intensity decreases as the cells enlarge. The maximum basophilia is encountered in the relatively non-hypertrophied cells of the decidua parietalis. In the progestational uterus the scant cytoplasm of all the endometrial connective tissue cells contains a homogenous basophilic wash which is generally less conspicuous than that of the decidua parietalis cells of comparable size.

In the paraplacental cells the basophilia is diffuse and dust-like in earlier stages, becoming somewhat coarser in later stages, but with no overall increase in intensity. The basophilia of the cells of the deeper basalis is more uniformly distributed as a fine stipple in the slightly vacuolated cytoplasm. No "grain" is visible in the intense bluish purple basophilia characterizing the cells of the parietal decidua late in gestation.

The capillaries of the endometrium, especially those at the future implantation site in the antimesometrial wall of the uterus, display prominent progestational modifications, which consist chiefly of proliferation and dilatation (Wimsatt, '44). Most of the arterial capillaries leading in from the outer segments of the uterine wall branch but little until they arrive

beneath the lining epithelium of the uterus, where they form a dense capillary plexus. This plexus is engulfed by the syncytial trophoblast at the time of implantation, and very early, therefore, provides a favorable means of transfer of nutrients to the growing blastocyst. During progestation the cytoplasm of the endometrial endothelium displays a rather intense homogeneous basophilia, a tinctorial reaction that disappears from typical endothelium during the period of implantation. In the early post-implantation period the emergent venous capillaries of the placenta display a marked endothelial hypertrophy in that part of their length which lies within the narrow paraplacental decidual layer. This "swollen endothelium" disappears before mid-gestation, but during the time that it persists the low cuboidal cells are characterized by a uniform cytoplasmic basophilia of moderate intensity.

Membranous chorion. The membranous chorion undercuts the placental disc and is fused with its deep surface (fig. 1). The surface of the chorion is everywhere in contact with the uterus, and its outer trophoblastic cells vary in form from flattened (early) or cuboidal (late) mesometrially, to tall columnar in the antimesometrial subplacental segment of the chorion. Cytoplasmic basophilia is characteristic of all but the most primitive flattened trophoblastic cells, but during gestation varies in its distribution within the cells. In the earlier stages the dust-like particles of basophilic material are distributed uniformly through the cytoplasm of the cells, providing a dark background against which numerous vacuoles stand out sharply (fig. 4). As gestation advances the basophilia appears more homogeneous and progressively becomes restricted to the perinuclear cytoplasm of the basal segments of the trophoblastic cells. This localization is most evident in the subplacental chorion where the cells are taller, but characterizes equally well the columnar and cuboidal cells at the mesometrial pole of the chorionic vesicle (fig. 5); with occasional exceptions the apical cytoplasm of the trophoblastic cells is completely devoid of basophilic material. Only slight differences occur in the intensity of the basophilia during

gestation: they consist in an increased affinity for methylene blue in those regions of the cytoplasm that continued to be stained, a tendency which may find an explanation in the concentration of the ribose nucleoprotein in the basal cytoplasm.

Labyrinthine placenta. Two types of trophoblast, the cyto- and syncytiotrophoblast, are intimately associated during development in the discoidal placenta. The cytotrophoblast serves essentially as a germinal bed for the syncytium, and disappears completely at about mid-gestation: the syncytium alone will remain to participate in the formation of the definitive placental barrier. When implantation is completed the syncytium is disposed as a solid shell about the embryonic hemisphere of the ovum and rests upon a single layer of cuboidal cells, the "basal cytotrophoblast." Slightly later, solid cords of cells, the "cytotrophoblastic villi," grow from the basal cytotrophoblast into the overlying syncytium. Still later, these cords hollow out and are invaded by mesenchyme and allantoic vessels (Wimsatt, '45). The distribution of basophilic substances in the basal cytotrophoblast, cytotrophoblastic villi, and syncytial trophoblast shortly after implantation is shown in figure 3. The placenta illustrated is that of *Pipistrellus subflavus*, a species in which its early development resembles that of *Myotis lucifugus*. The most intensely basophilic material occurs in the cuboidal cells of the basal cytotrophoblast, and consists of coarse blue granules and filaments superimposed upon a uniform finely-granulated blue background, which contrasts markedly with the paler-staining syncytium. The intense cytoplasmic basophilia of the basal cytotrophoblast diminishes in the cells of the cytotrophoblastic villi. In the syncytial cytoplasm, especially around the nuclei, a pale but definite dust-like blue stippling occurs which is diffuse enough not to mask the eosinophilia of the underlying protein substrate. The admixture of pink and blue gives the syncytium a delicate purplish tint (not shown in the figure since the eosinophilia was not reproduced). Basophilia is very weak in wide areas of cytoplasm between the ill-defined clumps

of nuclei, and in the maternal endothelium that has been enclosed by the syncytium. Cytoplasmic basophilia is more conspicuous in the syncytium adjacent to the basal cytotrophoblast than elsewhere.

In a typical specimen near mid-gestation (6-mm embryo) the cytotrophoblastic elements have disappeared and the syncytial trophoblast is arranged in the form of labyrinthine tubules enclosing the maternal blood channels. The cytoplasmic basophilia of the syncytium is more pronounced than in the preceding stage. The basophilic materials consist of radially arranged striae, flocculent masses, and very fine particles which occupy the whole of the syncytial cytoplasm except a narrow intensely acidphilic strip adjoining the maternal blood space. The basophilic bodies are usually somewhat coarser and denser at the side of the labyrinth nearer the basalis.

Near the end of gestation the organization of the placental disc is similar to the preceding stage, but the syncytial tubules are larger, more tortuous and more numerous. Figure 6 shows the distribution of basophilic materials in the syncytial trophoblast of a fetus of 18 mm. The staining is more intense than at earlier stages, but the basophilic materials are more restricted in distribution, being confined largely to the central region of the cytoplasmic mass. As a result the cytoplasm of both the maternal and the fetal sides of the syncytium is markedly acidophilic, the pink band on the fetal side being less constant and less regular in width than that on the maternal side. The central basophilic zone widens around the nuclei. At this stage the cytoplasm of the mesenchymal cells (fig. 6) occasionally shows traces of a basophilic wash. Basophilia is not evident in these elements in earlier stages. The effect of ribonuclease on the basophilia of the syncytium is shown in figure 7.

Wislocki and Dempsey ('45) were able to demonstrate that in man the nucleoli of trophoblastic cells possess a basophilic component that can be extinguished by ribonuclease, but I have been unable to demonstrate this effect in the bat.

Yolk sac. In the bilaminar blastocyst the primitive yolk sac consists of a single layer of flattened entodermal cells which is adherent to the trophoblast. During implantation the extension of the extra-embryonic mesoblast forces these two layers apart, and as blood islands develop in the mesoblast the adjoining entodermal cells lose their flattened shape and become enboidal. Eventually the area vasculosa covers nearly all of the yolk sac, and most of the entodermal cells correspondingly hypertrophy. Eventually, also, the extension of the exocoelom severs most of the vascularized yolk sac (splanchnopleure) from its connection with the chorion. Once this has occurred a columnar epithelium is formed from the splanchnic mesoblast adjoining the exocoelom, and in its definitive condition the free segment of the yolk sac is composed of two columnar epithelia, joined by a narrow strip of connective tissue which contains the vitelline vessels (figs. 1, 2, 23).

In the primitive flattened state the entodermal cells display little or no cytoplasmic basophilia, but when they begin to hypertrophy they accumulate a marked basophilic wash which is most intense in the subnuclear, basal cytoplasm. Coarser blue granules may be irregularly present. Even in the youngest hypertrophied cells minute unstained vacuoles may be seen in the basophilic cytoplasm. By mid-gestation (6-mm embryo) the columnar entodermal cells are filled with coarser blue-staining masses, and the fine blue wash of earlier stages is less evident (fig. 9). The nucleus is situated apically and the subnuclear cytoplasm contains a number of dark blue basal striations. Above the nucleus granular basophilic bodies are present. Near the end of gestation (stage of 18-mm fetus) the basophilia has become even more localized, being concentrated around the nucleus in the apical cytoplasm of the entodermal cells (fig. 10). The diffuse blue wash of earlier stages has disappeared, unmasking the eosinophilia of the protein substrate.

The columnar mesothelial cells of the exocoelomic surface of the yolk sac show at all stages less intense basophilic staining

than the entodermal cells (figs. 9 and 10), and it is usually most evident in the basal cytoplasm.

Amnion. The flattened cells of the amniotic ectoderm occasionally show the presence of a small amount of ribose nucleoprotein in the form of minute perinuclear basophilic granules.

The distribution of glycogen

Glycogen is widely distributed in the placenta of the bat. Although it is most abundant in the paraplacental layer of the decidua basalis and in the yolk sac, it occurs in significant quantities at different stages in many other endometrial and fetal tissues.

Endometrium. The accumulation of glycogen in easily detectable quantities in the glandular epithelium of the uterus constantly precedes glandular degeneration, for glycogen is almost completely absent in this epithelium during progestation when the cells pass through their maximum proliferative and secretory phases, but accumulates rapidly in the cells of glands approaching degeneration (figs. 15 and 17). The glycolytic cycle in the surface epithelium is less clear, for the epithelium at the implantation site is destroyed in a very short time, and the critical stage is not represented in my series. It is certain that, with the exception of a minute droplet in an occasional cell, glycogen is not present in the surface epithelium during the progestational period and the homogeneous non-vacuolated condition of the cytoplasm of specimens at the critical stage prepared by other methods strongly suggests that at the implantation site, at least, these cells are destroyed by the trophoblast before glycogenesis can occur. (The distribution of glycogen in the epithelial elements of the endometrium in other mammals at this time is reviewed by Dawson and Kosters, '44 and Wislocki and Dempsey, '45.)

The glands, on the other hand, lie deeper in the endometrium and are protected for a time from the direct destructive influence of the trophoblast. In response to stretching, compression, and the resulting localized ischemia of the uterine wall,

produced by expansion of the chorionic sac, the glands begin to degenerate (Wimsatt, '45). The accumulation of glycogen within the cells may represent a response to a reduction in circulatory efficiency, since it is well known that regions of sluggish metabolism are frequently characterized by glycogen deposition (Dempsey and Wislocki, '44). In the decidua basalis the deposition of glycogen begins simultaneously in all of the glands, but in the much thinner decidua parietalis deposition occurs first in the most superficial glands and gradually takes place in those more deeply situated. Once glycogen appears in the epithelial cells it soon shows also, in the form of a pale wash, in the gland lumen, and mesometrially, where the glands open, in the uterine lumen. It is not definitely known whether any of the glycogen in the gland lumen has been secreted; it seems more likely that most of it results from the disintegration of glycogen-containing cells. The glycogen in the uterine lumen mesometrially is undoubtedly derived from the glands and from glycogen-containing decidual cells that are being destroyed by the trophoblast in this area (figs. 17 and 21). All of the glycogen in the degenerating glands is ultimately absorbed by the trophoblast (figs 18 and 21).

Before leaving the epithelial components of the uterus it is necessary to consider an interesting development, apparently undescribed heretofore, which occurs during progestation in the epithelium of the intramural segment of the oviduct and of the tubal papilla marking the tubo-uterine junction. In the early post-ovulatory period the epithelial cells hypertrophy and become tall and columnar. Slightly later they become increasingly achromatic and display a peculiar vacuolation (fig. 25), in remarkable contrast to the remainder of the oviducal epithelium. (Seasonal histological changes in the oviduct of the bat have been described in some detail by Reeder, '39.) I have found that the unusual appearance of the oviducal epithelium is produced by the accumulation of massive quantities of glycogen in the epithelial cells (fig. 26), while the remainder of the oviducal epithelium contains less

of it. Not infrequently patches or individual cells of the glycogen-laden oviducal epithelium are found in the uterine lumen in late progestational stages, where they undoubtedly break down and may be absorbed by the ovum, perhaps providing a special source of energy for the unattached blastocyst.

Before implantation, glycogen is practically absent in the connective tissue cells of the endometrium, but after implantation it accumulates widely in the connective tissue. It appears nowhere as abundantly, however, as in the paraplacental layer of the decidua basalis (fig. 1). The cells of this layer, from the early post-implantation period when it first becomes distinguishable, to late gestation when it ultimately disappears, are remarkably distinct from all other decidual cells by virtue of their large size and coarse vacuolation. It was shown in an earlier paper (Wimsatt, '48) that these decidual cells contain relatively little lipid, their vacuolated condition presumably indicating the presence of some other substance. As shown in figures 15 and 16, this substance is glycogen, and the typical paraplacental cell at every stage of gestation is loaded with it. There is a tendency in many specimens for the slightly smaller, more superficial cells adjoining the placental disc to contain somewhat less glycogen than the larger, deeper cells. It may be more than coincidence that these cells also contain more lipid than the deeper cells (Wimsatt, '48). It should be emphasized, however, that the differences in glycogen content in the paraplacental decidual cells are only relative; compared with decidual cells elsewhere in the endometrium the superficial cells are heavily laden. Thus the placental disc during most of gestation is joined to the uterus exclusively by means of a layer of highly specialized glycogen-bearing endometrial cells which is penetrated by the maternal vessels entering and leaving the placenta.

The narrow zone of necrotic tissue that intervenes between the paraplacental layer and the deeper decidua basalis (fig. 1), and which is formed principally by the degeneration of the more superficial elements of the deeper zone (Wimsatt,

'48), contains only insignificant traces of glycogen at all stages of gestation.

The second largest accumulations of glycogen in the decidua occur in the hypertrophied cells of the deeper decidua basalis on each side of the mid-line (uterus viewed in cross section as in fig. 1). This zone comprises the greater thickness of the basalis in early stages (Wimsatt, '44), but the constant degeneration of the more superficial cells medially, and the histolytic activities of chorionic protrusions which invade it via the uterine glands laterally (Wimsatt, '45), gradually reduce it, and ultimately destroy it completely. Once the cells of this layer begin to hypertrophy (shortly after implantation), the deposition of glycogen ensues, and throughout gestation the majority of the cells reveal stainable glycogen in the form of minute cytoplasmic globules. There is considerable variation in the amounts of glycogen present in individual cells, but none of them contains relatively so much as a typical cell of the paraplacental layer.

Most of the connective tissue cells of the parietal decidua contain small cytoplasmic deposits of glycogen, these being noticeably heavier in the cells nearer the chorion, which is everywhere in contact with the parietalis (figs. 1, 18 and 20).

As gestation advances the large vessels penetrating the basalis and conveying maternal blood to the placental disc develop thickened perivascular sheaths of cellular connective tissue. In the outer layers of the sheaths glycogen becomes fairly abundant.

Membranous chorion. Glycogen is principally confined to the trophoblastic cells of the chorion, where it has been demonstrated in all stages from an implanting blastocyst to a 16-mm fetus. The subplacental segment of the membranous chorion of a specimen at term (18-20-mm fetus) contains no stainable glycogen. During most of gestation the amount of glycogen in the trophoblastic cells of this segment far exceeds that found in any of the trophoblastic constituents of the discoidal placenta at any time.

At the stage of implantation glycogen is conspicuous in the trophoblastic cells of the bilaminar omphalopleure, especially at the abembryonic pole of the blastocyst mesometrially, where the adjoining endometrium is undergoing erosion (figs. 17 and 18). The heaviest accumulations of glycogen in the trophoblast seem invariably to adjoin regions of endometrium where the cells are richer in glycogen than in surrounding areas. Similarly, endometrial areas with little glycogen are faced by chorionic cells of relatively lower glycogen content. In a slightly older specimen (1-2-mm embryo) the most conspicuous accumulations of glycogen lie in the trophoblastic cells of the equatorial region of the blastocyst near the margin of the placental disc, which is fairly well developed at this stage. Again the glycogen-laden cells adjoin a restricted area of disintegrating endometrium relatively high in glycogen content. These relationships suggest that the glycogen of the chorion is derived largely by absorption from the disintegrating endometrial cells.

Glycogen remains abundant in the trophoblastic cells of the membranous chorion throughout the first two-thirds or three-quarters of gestation (figs. 19, 20), that is, during the time that is required for the trophoblast to complete the destruction and absorption of all of the parietal, and most of the basal decidua. The specialized and temporary chorionic protrusions which penetrate deeply into the basalis (the so-called "villous chorio-allantoic placenta," Wimsatt, '45) during the middle third of pregnancy contain glycogen in equal measure with the remainder of the chorion, for they perform a similar function, the destruction and absorption of the endometrial tissue, which in the deeper basalis could not be attacked early and directly except by their agency. In the later stages of gestation a marked diminution in the quantity of glycogen in the trophoblast occurs, roughly coinciding with the reduction in its histolytic and absorptive activities, for the trophoblast has by now "eaten" its way to the myometrium, beyond which

it will not go. In the subplacental segment of the chorion glycogen continues to be found in relatively late stages in small groups of trophoblastic cells, especially in those which here and there form narrow incursions into the uterine tissue (fig. 20), but the quantities in the individual cells are small, and the majority of cells contain no stainable glycogen whatever. Mesometrially the chorionic cells containing traces of glycogen are even more scattered.

Because fixatives alter the normal distribution of glycogen in the cytoplasm, the localization of the substance in the cells will not be discussed, for it is felt that the intracellular distributions observed in fixed tissues do not in most instances reflect the true distribution during life.

Labyrinthine placenta. With the exception of the maternal blood which circulates within it the definitive placental disc is comprised wholly of fetal tissues, the syncytial trophoblast, the mesenchymal stroma and the fetal vessels. In early stages it contains a cytotrophoblast which is used up in the process of forming additional syncytium, and maternal endothelium, which quickly disappears. Among these constituents stainable glycogen occurs only in the cytotrophoblast, and in late pregnancy, in occasional mesenchymal cells. Minute glycogen deposits are found during implantation in only a small proportion of the cells of the "basal cytotrophoblast," which adjoins the syncytial trophoblast, in marked contrast to the relatively heavy deposits observed in the trophoblastic cells of the bilaminar omphalopleure that are directly in contact with the glycogen-rich decidua mesometrially. The number of glycogen-bearing cells very gradually increases in the basal cytotrophoblast during the period of growth of the solid "cytotrophoblastic villi" (Wimsatt, '45), and, in many of the latter, cells are found which contain glycogen in small quantities (fig. 16). The number of cells involved and the quantity of glycogen in individual cells gradually increases with further development and reaches a maximum shortly before the

cytotrophoblast disappears at mid-gestation (fig. 24). The glycogen-containing cells seem to be more numerous in the deeper portions of the placenta, i.e., nearer the decidua basalis.

The glycogen-bearing mesenchymal cells are too inconstant to have any obvious significance.

Finally, it should be emphatically stated that the predominant tissue of the placental disc, the syncytial trophoblast, at no period of gestation displays the slightest trace of glycogen.

Yolk sac. The yolk sac and the paraplacental decidua were earlier said to have a heavier glycogen content than any other placental tissue. The columnar mesothelium which covers the exocoelomic surface of the free segment of the yolk sac (figs. 21, 22, and 23), is particularly rich in glycogen at all stages of pregnancy, but it is found also in the entoderm in significant quantities (figs. 21, 22 and 23).

Glycogen first appears in the entodermal cells at implantation, when these cells are transforming from their primitive flattened condition to a cuboidal or columnar form. At this time not all cells contain it and it is resolvable only under the high dry and oil-immersion objectives (Bauer-Feulgen stain). As development proceeds more cells acquire glycogen and the deposits become larger (fig. 21). The quantity of glycogen in the entodermal cells, however, never approaches that which ultimately accumulates in the mesothelial cells—it remains more finely divided, and higher magnifications are required for its resolution. In a specimen near term (18–20-mm fetus) glycogen is less abundant in the entoderm than at earlier stages.

During the earlier stages of development no stainable material is observed in the cavity of the yolk sac, but after the 15-mm stage the small amount of coagulate in the cavity acquires a pale lavender hue following the application of the Bauer-Feulgen technique. This staining is lessened, but not eliminated in saliva-digested control slides.

Glycogen deposits are found irregularly also in the mesenchymal cells separating the two epithelia of the yolk sac

(fig. 21), and in limited quantities in the avascular sheet of mesoderm (still uninvaded by the exocoelom) which in earlier stages pushes between the entoderm and trophoblast (Wimsatt, '45).

Amnion. Glycogen occurs only in the amniotic ectoderm, which remains throughout gestation a single layer of very flattened and structurally undifferentiated cells. It was not observed (Bauer-Feulgen preparations) in the amniotic ectoderm during early pregnancy, but it is abundant in the cells of a specimen near mid-gestation (6-mm embryo) as shown in figure 21. In a 10-mm specimen only a faint reaction is here and there observed in the amnion. In specimens of 15, 16, and 18-20 mm the amnion is negative for glycogen.

The distribution of phosphatases

Twelve gravid uteri of different ages were available for histochemical analysis of phosphatase activity.² Sections were incubated in solutions of Na- β -glycerophosphate, ribose nucleic acid, fructose 1-6 diphosphate, adenylic acid and lecithin

²Little is known concerning the length of time phosphatase is preserved in embedded tissues, but the few observations available indicate a great variability. The subject is briefly discussed by Lison ('48). According to Lison, Danielli obtained typical results in pieces of kidney that had been kept in the cold for a year. On the other hand after the tissues were cut, phosphatase activity rapidly declined within them for no known reason. Lison describes the disappearance of phosphatase activity from "... des blocs renfermant une phosphatase tres active ..." at the end of two months at ordinary temperatures, and in non-deparaffinized sections the reaction became negative at the end of 12 days. Lison does not mention the tissues he used. According to Danielli the weakening of the reaction with time, or as a consequence of the technical procedures employed is everywhere proportional, so that the distribution of phosphatase remains always the same.

In the present study sections were cut and stained for phosphatase immediately after the original embedding in paraffin, and again after the lapse of from one to two years. In the majority of instances the passage of time had no effect on either the localization or intensity of the phosphatase reactions. This, together with the fact that both the earlier and later runs were checked by means of the usual controls, indicates that in the placental tissues of the bat phosphatase may be preserved in the block for as long as two years.

at pH 9.4, 7.0³ and 4.7. The standard incubation times at the 3 pH levels were 4, 48 and 72 hours respectively, except that in the alkaline range sections were incubated in the lecithin solution for 24 hours. Only alkaline glycerophosphatase⁴ was investigated up to the 5-mm stage, which occurs slightly before mid-gestation. The diversity of techniques employed permitted numerous details to be observed, but because of lack of space the description will be limited wherever possible to the more general aspects of phosphatase distribution in the various tissues of the placenta.

Endometrium

- *Epithelium.* Alkaline glycerophosphatase is abundant in the epithelium of the uterus and glands during progestation (fig. 27), being especially concentrated in the supranuclear cytoplasm. It is present also in the glandular and uterine lumens and in the nuclei of the epithelial cells, where it is localized in the nuclear membranes, chromatin particles, and nucleoli. Segments of the glands persist mesometrially and may, before they ultimately disappear, be the source of the surface epithelium which in late stages of gestation is reconstituted in this area. Shortly after implantation alkaline glycerophosphatase activity rapidly diminishes in the glands.

³ It is rather difficult to determine the validity of some of the reactions obtained at neutral pH, for in many of the preparations all tissue elements displayed a moderate brown coloration. It is possible in such instances that one or the other of the chemical intermediates formed in the course of the "indirect" reaction has been adsorbed by the various tissue constituents, so that when the reactions are completed the adsorbed substances may produce a false image of phosphatase distribution. (This factor is discussed at some length by Lison, '48.) In the accounts to be presented of phosphatase activity at neutral pH such general "background" reactions will be ignored, and only those reactions which by virtue of their constancy are believed to be valid will be described.

⁴ The use of such compound terms as "alkaline glycerophosphatase," "neutral nucleic acid phosphatase," "acid hexose diphosphatase," etc., is not intended to indicate that each of these is regarded as a distinct and separate enzyme; the terms are merely descriptive, and their use constitutes the most convenient way of indicating the pH and substrate environments in which phosphatase activity was sought.

This activity eventually disappears completely from the fundi and deeper portions of the ducts, but persists in the cells nearer the surface and is present also in the reconstituted surface epithelium mesometrially. The reaction is principally confined to the terminal zone of the apical cytoplasm, and occasionally appears in the nuclei of the positive cells. With the nucleic acid substrate phosphatase activity is more intense in the epithelium (fig. 28), and with fructose diphosphate, less intense, than with glycerophosphate, but the intracellular localization of the enzyme remains the same. The nuclear reactions are more constant with nucleic acid. A weak cytoplasmic reaction occurs in the superficial epithelial cells following adenylic acid, but the nuclei, even of many of the deeper cells, stain intensely. There is no evidence of alkaline⁹ lecithinase activity in the epithelial elements at any stage of gestation after 24 hours of incubation.

At pH 7.0 negative results were obtained with all substrates, except that traces of nucleic acid phosphatase were observed in the epithelial nuclei during the third quarter of gestation. Phosphatase activity at neutral pH was not investigated in the epithelium in late gestation.

At pH 4.7 the epithelial cytoplasm during the second half of gestation (not observed in first half) is completely negative with all substrates, but the nuclei show a variable reaction with glycerophosphate, nucleic acid and adenylic acid. The nuclear reaction with glycerophosphate and nucleic acid diminishes somewhat in later stages, whereas that with adenylic acid appears to increase, the most intense reactions being observed in a near-term specimen. In a single specimen at mid-gestation (6-mm embryo) an intense acid phosphatase reaction was observed in the lumens of two deeply placed glands of the decidua parietalis; there was no reaction in the adjoining cells of the glands.

Decidua. Phosphatase activity varies considerably in different regions of the decidua during gestation. Alkaline glycerophosphatase is present until shortly after implantation in the nuclei of the connective tissue cells (fig. 27), but only

irregularly thereafter. Only traces of the enzyme are found in the cytoplasm before implantation, but the amount rapidly increases afterward. Alkaline glycerophosphatase activity remains high in the cells and stroma of the decidua parietalis mesometrially, but after the initial increase declines in the cells of the deep decidua basalis. With nucleic acid and fructose diphosphate corresponding reactions are observed, but with greater and lesser intensity respectively than with glycerophosphate. The nuclei are more frequently stained with nucleic acid, and almost never with fructose diphosphate. With adenylic acid at pH 9.4 a conspicuous nuclear reaction characterizes the decidual cells throughout the second half of gestation, being in later stages perhaps most intense mesometrially. Cytoplasmic and stromal reactions are moderate at mid-gestation but disappear later. Except for a faint staining of the nuclei of the deepest basalis cells at the 5-mm stage no alkaline lecithinase activity was observed in the deciduae.

At pH 7.0 traces of glycerophosphatase occur in the decidual cells and stroma of the deep basalis, and in the nuclei of the decidual cells. In the mesometrial region of the parietal decidua the reactions are stronger than in the basalis. The reaction is comparable following nucleic acid, but more intense with fructose diphosphate. With adenylic acid only the nuclei give a moderate reaction at neutral pH, and lecithinase is completely absent. At pH 4.7 traces of phosphatase are limited to the decidual nuclei in the glycerophosphate and nucleic acid preparations.

In the cells of the paraplacental decidua phosphatase activity is limited to a moderate nuclear reaction with nucleic acid at pH 9.4, adenylic acid at pH 7.0, and to a faint nuclear reaction with nucleic acid at pH 4.7. No cytoplasmic or stromal reaction was observed in the paraplacental decidua with any substrate at any level of pH.

Endothelium. Phosphatase activity was observed in the endothelium of the endometrial vessels in only a few instances. Alkaline glycerophosphatase occurs consistently only in the capillaries that have been enclosed by the syncytial tropho-

blast during implantation (fig. 33). Observations concerning phosphatase activity with other substrates, and at neutral and acid pH were not made during the brief period that this engulfed endothelium persists. The "swollen endothelium" in the paraplacental decidua (see p. 69) was likewise examined only for alkaline glycerophosphatase and was found to be negative (fig. 33). Fortuitous reactions were observed in later gestation in endothelium immediately associated with decidual tissue containing the enzyme, and not elsewhere in the same sections; the positive material may represent enzyme that has diffused into the endothelium from the surrounding tissue, possibly as a result of faulty fixation. A similar distribution of enzyme is observed with nucleic acid and fructose diphosphate. No enzymatic activity was observed in the maternal endothelium at pH 7.0 and 4.7.

Myometrium

Phosphatase activity is pronounced in the myometrium during most of gestation. Alkaline glycerophosphatase is not apparent before implantation (fig. 27), but it appears soon afterward and continues with undiminished intensity for the remainder of gestation (figs. 29, 30 and 31). The enzyme occurs in both the smooth muscle fibers and the intervening fibroblastic cells. In muscle the nuclear reaction is faint, but the cytoplasmic reaction may be intense and appears to be most concentrated peripherally (fig. 31). Because of their density, it is not possible to determine with certainty whether the peripheral concentrations are actually within the fibers or just outside them. In favorable sections a concentration of phosphatase in the myofibrils is apparent. Alkaline glycerophosphatase activity is often pronounced in the intermuscular fibroblastic cells, and tends to fluctuate in intensity with the enzymatic activity of the muscle fibers. Similar reactions in both muscle cells and fibroblasts are given with nucleic acid and fructose diphosphate (figs. 29, 30 and 31), the former giving the greatest reaction of all. A less intense muscular

reaction occurs with adenylic acid, and with lecithin only an irregular and weak reaction was observed.

At pH 7.0 phosphatase activity is apparent only in the muscle with all substrates, but the staining is noticeably less intense than at pH 9.4, and the strongest reactions seem to follow fructose diphosphate rather than nucleic acid. At pH 4.7 a trace of glycerophosphatase occurs in the muscle only of a specimen near term, and the muscle and fibroblasts are completely negative with the other substrates.

Membranous chorion

Although phosphatases occur in the trophoblastic cells of the membranous chorion during later stages of gestation, their patchy distribution, and their tendency to occur only in those cells which adjoin endometrial zones containing phosphatase suggest that most of the chorionic enzymes, are not formed endogenously. Phosphatases are most constantly present in the chorionic cells adjoining the phosphatase-secreting uterine epithelial cells mesometrially (cf., p. 82), in the region, it may be recalled, where endometrial phosphatase activity is most pronounced in late gestation. The enzymes are usually concentrated in the apical cytoplasm, and strongly positive material is often adherent to the surfaces of the cells. The entire histological picture strongly suggests that the enzymes of the trophoblast, particularly those in the cytoplasm, have been absorbed from the adjoining endometrial elements. Mild phosphatase activity is observed in the mesometrial chorion at pH 9.4 with glycerophosphate, nucleic acid, fructose diphosphate and adenylic acid. With lecithin the trophoblastic cytoplasm is negative, and the trophoblastic nuclei are stained only sporadically at this pH.

At pH 7.0 the trophoblastic nuclei mesometrially give a moderate reaction with glycerophosphate, but with adenylic acid an intense nuclear reaction is given by all of the trophoblastic cells. A cytoplasmic reaction which is principally confined to the trophoblastic cells mesometrially is given with nucleic acid. With fructose diphosphate a patchy, but intense

reaction is given in all regions of the chorion, and with adenylic acid a faint reaction occurs in the subplacental chorion and a much stronger one mesometrially. No reaction occurs with lecithin.

At pH 4.7 no glycerophosphatase or lecithinase activity was observed, but mild nuclear and cytoplasmic reactions occurred with nucleic acid, a patchy nuclear reaction was observed with fructose diphosphate, and adenylic acid produced an intense general nuclear reaction.

The universal staining of the nuclei in the adenylic acid preparations at pH 7.0 and 4.7 probably attests the endogenous origin of at least one chorionic enzyme.

Labyrinthine placenta

Phosphatase activity in the labyrinthine placenta is principally confined to the syncytial trophoblast, although irregular nuclear reactions are also encountered in the fetal mesenchymal cells and endothelium. Enzymatic activity in the embryonic tissues of *Myotis* was not investigated until early in the post-implantation period (embryo of 2 mm). It is probably pertinent, however, that alkaline glycerophosphatase is absent in the tissues of an implanting blastocyst of *Pipistrellus subflavus*, in which the early development resembles that of *Myotis*.

In *Myotis* at the 2-mm stage the basal cytotrophoblast and the membranous trophoblast are devoid of phosphatase, but the nuclei of the cells nearer the apices of the cytotrophoblastic villi, and the cytoplasm of those cells adjoining the syncytial trophoblast, contain traces of alkaline glycerophosphatase (fig. 33). The syncytial trophoblast reveals a more constant reaction, consisting of a faint central wash, which, at the maternal and mesenchymal borders of the syncytium, deepens, indicating greater enzymatic activity in the peripheral cytoplasm. The nuclei of the syncytium are strongly positive.

In later stages variations are observed in the distribution of alkaline phosphatase within the cytoplasm of the syncytial

trophoblast when different substrates are used. With glycerophosphate and fructose diphosphate (and with lecithin in one placenta at mid-gestation) a narrow band of cytoplasm adjoining the maternal blood spaces displays a much more intense reaction than the remainder of the cytoplasm (figs. 34, 35). This narrow zone of greatest phosphatase activity corresponds to the outer acidophilic strip of eosin-methylene blue preparations (fig. 6) and the differentially stained (by acid dyes) outer zone of the syncytium following the Azan and Masson trichrome procedures (Wimsatt, '45). With ribose nucleic acid a stronger general reaction is given in the syncytium than with the foregoing substrates, but an outer zone of concentration is absent at all stages examined (figs. 29, 30 and 36). With adenylic acid and lecithin a very faint reaction occurs in the syncytium, but with the single exception of the specimen treated with lecithin at mid-gestation, no outer concentration of enzyme is detectable.

In marked contrast to what occurs in other mammalian placentas (Dempsey and Wislocki, '47) cytoplasmic alkaline glycerophosphatase activity gradually diminishes in the syncytium of the bat as gestation progresses, and by the latest stages examined (near term) enzyme activity is greatly reduced. With nucleic acid, on the other hand, a decrease in activity is much less evident (figs. 29, 30 and 36). Hard ('46) and Dempsey and Wislocki ('47) report a decrease of glycerophosphatase in the syncytium of the guinea pig during the last quarter of gestation. Phosphatase activity is more lasting in the syncytial nuclei. Whereas the nuclei are negative at all later stages with glycerophosphate (c.f. glycerophosphatase activity of nuclei at 2-mm stage, fig. 33), and only faintly positive at the 18-mm stage with fructose diphosphate, a weak reaction is given with nucleic acid at mid-gestation and increases in later stages (figs. 30 and 36). The nuclei stain faintly at all stages with adenylic acid, and with lecithin a very faint nuclear reaction was observed only in one specimen near mid-gestation.

Alkaline phosphatase activity was observed in the nuclei of the fetal mesenchymal cells only after incubation in nucleic acid.

The fetal endothelium of the placental disc is negative with all substrates.

At pH 7.0 positive cytoplasmic reactions in the syncytial trophoblast follow incubation in glycerophosphate, nucleic acid, fructose diphosphate and adenylic acid. Glycerophosphatase and adenylic acid phosphatase are least conspicuous. An outer zone of greater enzymatic concentration was observed in only one specimen (6-mm stage), following glycerophosphate. Unlike alkaline phosphatase, neutral phosphatase does not markedly diminish during pregnancy (see, however, footnote 3, p. 81). The syncytial nuclei are positive with all substrates except lecithin, the reaction increasing during gestation with glycerophosphate and nucleic acid, and remaining essentially unchanged with fructose diphosphate and adenylic acid. The most intense nuclear staining follows the use of adenylic acid. The nuclei of the mesenchymal cells react sharply in late stages with glycerophosphate and nucleic acid, and throughout the latter half of pregnancy with adenylic acid. They are uniformly negative with lecithin. Closely adjoining the mesenchymal surface of the syncytial trophoblast at the 6- and 10-mm stages are flattened cells which may represent cytotrophoblastic remnants (Wimsatt, '45) or adherent mesenchymal cells. Their nuclei show marked phosphatase activity at neutral pH with nucleic acid, fructose diphosphate and adenylic acid. In a near-term placenta at neutral pH the fetal endothelium displayed rather marked fructose diphosphatase activity which was most intense in the larger vessels nearer the fetal surface of the placenta. The endothelium was negative with the other substrates.

At pH 4.7, phosphatase activity is largely confined to the nuclei in the labyrinthine placenta. With glycerophosphate, fructose diphosphate and lecithin no activity whatever was observed, and with adenylic acid a positive reaction was found only in the syncytial nuclei of a near-term placenta.

With nucleic acid, on the other hand, a faint to moderate reaction characterized the cytoplasm of the syncytium and the nuclei of the fetal mesenchymal cells in a near-term placenta. A moderate reaction was observed in the fetal endothelial nuclei at mid-gestation, but not later.

Yolk sac

The distribution of phosphatases in the yolk sac is less consistent than in other embryonic tissues. Completely negative results were obtained with lecithin at every pH level. Only alkaline glycerophosphatase was tested for in the first half of gestation, and the entodermal cells were completely negative. The first positive reaction in the yolk sac was observed in the embryonic stage of 9 mm, wherein alkaline glycerophosphatase was observed in small to moderate amounts in the cytoplasm of the entoderm, the mesothelium, and the endothelium of the vitelline vessels. At the 18-mm stage traces of glycerophosphatase are found apically in some of the mesothelial cells. No reaction occurs with nucleic acid at mid-pregnancy, but at the 18-mm stage traces of alkaline nucleic acid phosphatase are found in the entodermal and mesothelial cells, the strongest reactions characterizing the nuclei (fig. 32). A similar distribution of enzyme is revealed by fructose diphosphate, except that near term the cytoplasmic reaction in the mesothelial cells is concentrated apically. No reaction occurs with adenylic acid at mid-gestation, but the entodermal, mesothelial and endothelial nuclei stain moderately in late gestation, and there is a faint apical reaction in the mesothelial cells.

At pH 7.0 positive phosphatase reactions were observed only with fructose diphosphate and adenylic acid. With the former substrate the entodermal cells at the embryonic stage of 10 mm reveal a faint cytoplasmic reaction which becomes more concentrated apically. At the same stage the entodermal and mesothelial nuclei give a strong reaction with adenylic acid. Neutral phosphatase was not investigated with these substrates in late gestation.

At pH 4.7 phosphatase activity was observed with glycerophosphate, nucleic acid and adenylic acid. With nucleic acid the entoderm in a near-term specimen gave a moderate cytoplasmic and nuclear reaction, and the mesothelium a weak nuclear reaction. With glycerophosphate weak nuclear reactions were observed in both epithelia at the same stage, and with adenylic acid an intense nuclear reaction characterized the entodermal, mesothelial, and vitelline endothelial cells.

Amnion

The flattened cells of the amniotic ectoderm contain phosphatase more often and in greater concentrations than any other embryonic tissue. Alkaline phosphatase is present at all stages of pregnancy and following all substrates. The reactions are most pronounced with nucleic acid (figs. 30 and 32) and glycerophosphate but intense activity is also observed with fructose diphosphate and somewhat less intense activity with adenylic acid. Alkaline lecithinase activity is weak. At pH 7.0 reactions are similar, but less intense with all substrates. There is a distinct tendency for the neutral phosphatase activity with all substrates to increase as gestation advances. At pH 4.7 a weak to moderate reaction occurs in the amnion with nucleic acid only. The reaction is greater in older specimens.

The distribution of lipase

Wachstein's ('45) modification of Gomori's ('45) histochemical test for lipase was applied to three placentas in representative stages of gestation. Positive reactions were obtained in control sections of pancreas and liver treated simultaneously with the sections of the placenta, but no lipase activity was observed in the placenta or fetal membranes. Dempsey and Wislocki ('47) were unable to demonstrate lipase activity in a human placenta at term.

Lipase has been detected biochemically in the human placenta (Needham, '31), and its failure to be revealed by the histochemical tests may be due to the loss of enzymatic

potency resulting from the technical procedures utilized, or from prolonged storage of the paraffin-embedded tissues before sectioning and staining. In two of the bat placentas a year elapsed—and in the third, two years—between the embedding of the material and the application of the procedure for lipase. It is probable that the human placenta material described by Dempsey and Wislocki was also on hand for some time before being stained, for these authors state that “Our experimental material consists of sections cut from selected blocks prepared for our previous studies of the placenta.” It is pertinent, however, that the liver and pancreas control sections run with the bat placenta were sectioned and mounted 6 months beforehand, and there was within them during that length of time no detectable diminution in either the distribution or intensity of lipase activity.

The Bodian protargol method in the placenta

It has been suggested that the Dawson and Barnett ('44) modification of the Bodian protargol reaction may reveal the presence of formed calcium deposits in placental tissues (Wislocki and Dempsey, '45), a suggestion based upon the observation that acid-soluble Bodian-positive granules are found at placental sites known to contain calcium. In order to determine the extent and nature of the placental reaction in the bat the Bodian procedure was applied to sections of 5 placentas of different ages (from implantation to the fetal stage of 15 mm), and variously preserved in Bodian's fixative no. 2, Bouin's fluid, and a mixture of absolute alcohol, picric acid and formalin. Control sections of material preserved in Bodian's fixative were treated 24 to 48 hours in 5% aqueous hydrochloric acid or acetic acid before being stained.

Among the fetal constituents of the placenta argyrophilia was observed only in the subplacental chorion and the cytoplasm of a smaller number of nucleated fetal erythrocytes. In the chorion the reaction is limited to a few granules which appear sporadically in the trophoblastic cells. Among the maternal constituents a positive silver reaction is observed

only in the necrotic zone of the decidua basalis. Here, argyrophilic granules of various sizes occur, most of them extracellular, but some are intracellular. Since all of these reactions are given on sections of material preserved in fixatives containing acid or extracted in acid solutions, it is concluded that in the bat the positive silver reaction does not indicate the presence of formed deposits of calcium salts as it does in other mammals.

Hematogenous pigments, characteristic of the placentas of other mammals, are scarce in the bat, being restricted to small deposits in the necrotic zone and sporadic granules in the subplacental trophoblastic cells. Since it is well known that the Bodian procedure blackens and intensifies such pigments (Wislocki, Deane and Dempsey, '46; Wislocki and Wimsatt, '47), it is possible that part of the argyrophilia in the necrotic zone and chorion represents the impregnation of pigment granules. The significance of the remaining argyrophilic granules, and of those in the nucleated erythrocytes is unknown.

The distribution of iron

The distribution of iron was studied in 12 placentas representing the entire period of pregnancy. The investigative procedures including the Turnbull blue reaction (Lison, '36), the Prussian blue reaction, and the Macallum reaction for "masked iron" (Bensley and Bensley, '38). Congruent results were obtained with all procedures, except that the Macallum test revealed some additional iron not demonstrated by the Turnbull blue or Prussian blue tests. In a few instances sections were treated with the "unmasking" acid alcohol solution of the Macallum procedure and then exposed to the Turnbull blue reagents: for some unknown reason a positive reaction was not evoked in sections so treated. Iron was revealed most abundantly with all of the tests in the mesenchymal stroma of the labyrinthine placenta. Sporadically, iron was also revealed by the Turnbull blue and Prussian blue reagents in the yolk-sac entoderm, the chorionic trophoblast

of a young implanted specimen, and the endometrial stroma during implantation; positive reactions were nearly always given with these tests by the fetal and maternal erythrocytes. With the Macallum test the intense reaction in the fetal stroma obtained with the other tests is greatly diminished in intensity, but faint traces of iron are revealed in the syncytial trophoblast, and in most nuclei, sites where it was not usually detectable by the other procedures. The following account will be concerned only with cytoplasmic and extracellular iron — the presence of Macallum-positive “organic” iron in the nuclei of all tissues may be assumed.

Endometrium. A positive reaction for iron was not observed in the glandular or surface epithelium of the uterus, either during progestation or gestation, nor was one obtained in the glandular or uterine lumens at any stage. Iron was present in the endometrial stroma of only one specimen, at the stage of implantation. It exists in the form of minute cytoplasmic granules in occasional, widely separated, connective tissue cells. The iron-containing cells are more numerous, but still sporadic, adjoining the capillaries of the subepithelial vascular plexus at the implantation site.

Membranous chorion. A positive reaction for iron was obtained in the trophoblast of the membranous chorion in only one, newly-implanted, ovum (2-mm embryo). The columnar trophoblastic cells of the abembryonic (mesometrial) pole of the ovum irregularly contain minute blue granules (Turnbull blue). The trophoblastic cells of all other specimens examined throughout pregnancy were completely negative. That iron may occur in small amounts at later stages, however, is indicated by the fact that extravasated maternal erythrocytes are irregularly ingested by the trophoblastic cells in small quantities (fig. 4), and iron is presumably released during their digestion.

Labyrinthine placenta. The distribution of iron in the mesenchymal stroma of the labyrinthine placenta is illustrated in figures 12 and 13. Attention is directed to three constant features of its distribution: (a) it is found predominantly in

the stroma of the deeper half of the placental disc, nearer the decidua basalis, (b) it is not generally distributed in the stroma, but occurs in patches, and (c) it is invariably most concentrated in the stromal elements immediately adjoining the fetal syncytium where it occurs both within and without the stromal cells, and often forms a continuous layer of iron between the syncytium and the deeper stroma. Iron was first observed in the mesenchymal stroma in the deeper half of the placenta at mid-gestation (6-mm embryo). Thereafter it gradually increases and is present in greatest amount in a specimen at the 16-mm stage, but it is still abundant at term. Although small deposits of iron are found in the stromal cells of the superficial half (nearer the fetal surface) of the placental disc in older placentas, continuous layers of iron in the stroma adjacent to the syncytium and resembling those of the deeper zone are only rarely present.

In an advanced implanting ovum of *Pipistrellus subflavus* iron is constantly present in the form of minute granules which are arranged about the most superficial blood channels invested by the syncytial trophoblast (fig. 11). It cannot be certainly demonstrated whether the maternal endothelium still persists; if it does then the bulk of the iron would lie in the endothelium, but if it does not, the iron lies within the most superficial zone of the syncytium. Some of the nuclei of the syncytial and cytotrophoblast also display a weak positive reaction in this specimen; all other elements, fetal and maternal, are negative.

The presence of "masked" iron in the syncytial trophoblast as revealed by the Macallum method is illustrated in figure 14. The reaction is so faint that the illumination (with daylight filter) must be very critically adjusted to bring out the pale blue tint indicative of the presence of iron. The blue color was observed, however, before it was found that a red Wratten filter (Eastman F filter no. 29) greatly intensified the fainter nuances and made them more visible. Figure 14 was drawn using this filter. The absence of stainable iron in the Turnbull blue and Prussian blue preparations, plus the fact that

paracarmine was used as a counterstain, causes the syncytial tubules to all but disappear in the red light in these preparations, in marked contrast to the intensification of the tubules by red light in the Macallum preparations.

The positive iron reaction in the mature fetal and maternal erythrocytes appears as a delicate and diffuse blue wash—there are no discrete granules. In the fetal erythroblasts, on the other hand, in addition to a general cytoplasmic staining, a more localized cytoplasmic reaction is given in the form of a variable number of minute dark blue granules (fig. 8).

Yolk sac. Cytoplasmic granules reacting positively for iron (Turnbull blue and Prussian blue) were present sporadically in the yolk-sac entoderm of but two specimens, one from the first half of gestation (2–3-mm embryo) and one from early in the second half (10-mm embryo). Iron was never observed elsewhere in the yolk sac at any stage of development.

The distribution of metachromatic substances

Metachromasia has been observed in the syncytial trophoblast of the human placenta (Wislocki, Bunting and Dempsey, '47) and in numerous connective tissue cells of the maternal placenta in several species of rodents (Asplund, Borell and Holmgren, '40) following the application of toluidine blue to sections of material fixed in 4% basic lead acetate. Recent studies have revealed that metachromatic staining can no longer be regarded as indicating the presence only of sulfuric acid esters of high molecular weight, as maintained by Lison ('36) and Holmgren ('40), for in addition to the sulfate-conjugated mucopolysaccharides, hyaluronic acid, nucleoproteins and other substances of unknown composition may stain metachromatically under various conditions (Wislocki, Bunting and Dempsey, '47; Dempsey, Bunting, Singer and Wislocki, '47). With proper control measures, however, the presence of certain definite chemical entities is revealed by metachromatic staining. For example, the abolishment of metachromatic staining in human placental syncytium that has been exposed to ribonuclease indicates that the metachromasia

is attributable to ribose nucleoprotein, and the similar eradication of metachromasia in the vitreous body and synovial fluid by the enzyme hyaluronidase suggests that hyaluronic acid is the metachromatic constituent in these tissues (Wislocki, Bunting and Dempsey, '47).

The detection of metachromatic substances in the placenta of other mammals made it appear desirable to search for these substances in the placenta of the bat. It is generally recognized that basic lead acetate is the most desirable fixative for the preservation of many metachromatic materials in tissue sections, but unfortunately, tissues so fixed were not available in this study. Accordingly the toluidine blue staining procedures were applied to tissues fixed in Zenker's, and Maximow's (Zenker-formalin) fluids and a mixture of absolute alcohol, picric acid and formalin. The absence in the bat of metachromasia in tissues which display it in other species does not necessarily mean that substances capable of staining metachromatically are not present, for different fixatives have variable effects on the selective staining of tissues by metachromatic dyes. Identical elements were stained metachromatically by the two different toluidine blue procedures (see p. 65) utilized in the present study.

In the placenta of the bat metachromasia was not observed in the syncytial trophoblast, despite the presence there of ribose nucleoprotein, nor, except as noted below, was it observed in any other fetal tissue. It was observed, however, at two situations in the maternal tissues—in the form of crowded cytoplasmic granules in certain connective tissue cells, and in the mucus and mucus-secreting cells of the uterine and cervical glands. The cervical glands were observed only in the last quarter of gestation, but at this time the metachromatically-stained mucus is copious in amount, and some lies in the uterine lumen adjacent to the abembryonic segment of the chorion (fig. 38). A faint tinting of the apical portions of the trophoblastic cells may indicate that some of the mucus is absorbed by the chorion. It is also of interest that the glandular mucus is stained intensely reddish by the Baner-

Feulgen method for glycogen, a reaction that is unaffected by salivary amylase (fig. 38). It is known that some sulfate-conjugated mucopolysaccharides are Bauer-positive, whereas hyaluronic acid, a sulfate-free mucopolysaccharide found in Wharton's jelly, synovial fluid and vitreous body is Bauer-negative (Wislocki, Bunting and Dempsey, '47). Hyaluronidase has no effect on the Bauer-Feulgen reaction and metachromatic staining of the glandular mucus of the bat, thereby ruling out hyaluronic acid as the chromophilic agent; a sulfate-conjugated mucopolysaccharide is the more logical possibility.

Cells containing metachromatic granules in their cytoplasm are present both in the myometrium and endometrium before implantation, but become increasingly restricted to the deepest portions of the endometrium and to the myometrium during gestation (fig. 37). They are numerous also in the loose connective tissue adjoining the mesometrium. The cells are most numerous and are concentrated in the myometrium of a specimen near mid-gestation (7-mm embryo), but no constant correlation was observed between the number of cells and the stage of gestation. They are still numerous in the myometrium of a uterus in the puerperium. There is no tendency for the cells to be more concentrated in the vicinity of blood vessels. The metachromatic granules are best preserved in the tissues fixed in a mixture of absolute alcohol, picric acid and formalin; following Zenker's and Maximow's fluids the metachromasia is as intense, but is relatively non-granular. The cells appear to be Bauer-negative. The granules are intensely basophilic when stained with methylene blue.

The tinctorial reactions and the position of the granulated cells in the uterus of the bat suggest possible affinities with the so-called "specific cells" (spezifische Zellen) of the gravid uterus in the rat and mouse (Asplund, Borell and Holmgren, '40). In these rodents cells with metachromatic granules are numerous in the uterus beneath the attachment of the discoidal placenta and are especially concentrated about the blood vessels. The cells appear early and gradually increase in

number and granule content, reaching a peak during late pregnancy (18 to 19 days in the rat), followed by a rapid decline, and ultimate disappearance after parturition. The "specific cells" rapidly degenerate and disappear following the experimental removal of the fetus and placenta, but not after the removal of the fetus alone. They may be induced to appear in the non-gravid uterus by the experimental production of placentomata. Because of these characteristics the cells are thought by Asplund, Borell and Holmgren to be identical with those constituting the so-called "myometrial gland" of other authors. Asplund, Borell and Holmgren do not interpret the "specific cells" of rodents as mast cells, which could also be identified in their preparations, but they do assign to them an analogous function — the production of a substance not identical with that in the mast granules, but having the same "heparin-effect." It is the belief of these authors that the secretion of the "specific cells" is discharged into the blood vessels which they surround and serves to prevent coagulation of the blood in the large sinus-like channels of the maternal labyrinth. The conclusion that the "specific cells" of rodents are not mast cells rests upon a combination of physiological, chemical and physical properties of the cells and their granules.

In the bat, on the other hand, the absence of any cells bearing a close histological resemblance to the "myometrial gland" cells of rodents, the failure of the metachromatic cells to be restricted to the region of placental attachment, the random distribution of the metachromatic cells, which bears no obvious relation to the distribution of the myometrial vessels, the basophilia of the cell granules (with methylene blue) coupled with their intense metachromaticity, the preservation of the metachromasia following Zenker and Zenker-formalin fixation, the failure of the cells to accumulate lipids and acidophilic plaques, and the histological resemblance of the cells to mast cells in other locations, suggests strongly that the myometrial cells are not homologous with the "specific cells" of rodents, but are in all probability true mast cells.

The distribution of proteinous globules in the visceral layer of the yolk sac

The presence of two columnar epithelia in the wall of the yolk sac is described on page 72, and illustrated in figures 1, 2, 9, 19, 21 and 23. The accumulation of chromophilic globules in the mesothelial cells (fig. 2) is briefly mentioned in two previous papers (Wimsatt, '45, '48), and was noted earlier in European bats by Branca ('23) — who also mentions finding them in the entodermal cells — and Gerard ('28). Similar droplets have long been known in the yolk-sac entoderm of rodents (Wislocki, Deane and Dempsey, '46, review the literature), and have recently been described in the trophoblastic cells of the chorionic fossae and areolae in the pig (Wislocki and Dempsey, '46b). The droplets have been universally described as "albuminous" or proteinous. They also bear a striking resemblance to the so-called "neurosecretory granules" of the nervous systems of many invertebrates and vertebrates (see review article of Scharrer and Scharrer, '45, and Palay, '45), the acidophilic inclusions of specific cells ("Schollenleukozyten") occurring in the intestinal mucosa of many mammals (Weill, '19), and certain acidophilic bodies associated with cellular degeneration at different sites in the developing embryo (Ernst, '26).

The globules do not appear in the yolk sac of the bat until the mesothelial cells have attained a cuboidal or columnar form during the second half of gestation. The number of cells containing granules progressively increases, but even at term fairly large groups of mesothelial cells have not acquired them. The granule-bearing cells are arranged in patches separated by areas of non-granulated cells. The globules are rarely found in isolated single cells. The development of granules in different specimens of corresponding ages varies considerably — the globules being numerous and widespread in one, and reduced in number and distribution in another. The granules themselves vary in size from 2 or 3 μ to the diameter of a fetal erythrocyte (fig. 2). The smaller ones

appear first, followed by the larger ones, but definitively, all sizes of granules may coexist in the same cell. When few in number the globules tend to accumulate in the basal cytoplasm (fig. 2 B), but when numerous they occur at random, above as well as below the nucleus (fig. 2 A).

The similar reactions of the globules in the bat, rodents and pig to various fixing and staining solutions suggests a related composition in the three species. In the bat the granules are not dissolved by alcohol, acetone, chloroform, hot

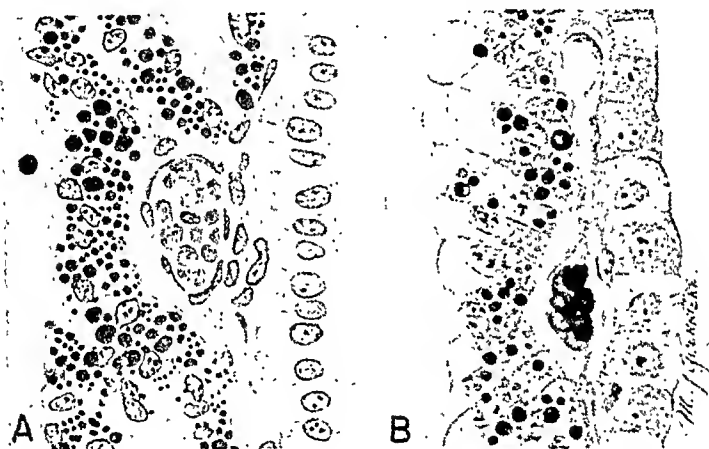


Fig. 2 Acidophilic proteinous inclusions in the mesodermal epithelium of the exocoelomic surface of the yolk sac as revealed by the Masson triacid stain (A) and Baker's ('46) pyridine extraction method (B). The inclusions were never observed in the entodermal cells, which line the yolk-sac cavity. In A a granule is anomalously present in the exocoelom, an artifact produced during the preparation of the section. A, $\times 728$; B, $\times 437$.

pyridine or dilute acids, and are retained after a variety of fixatives including 10% formalin, Zenker, Zenker-formalin, Bouin and Regaud. In general they display a marked affinity for acid dyes, staining orange, green or red with the Masson triacid mixture (fig. 2 A), blue to orange with the azan technique, reddish or purplish with eosin and methylene blue and pinkish with hematoxylin and eosin. Furthermore the drop-lets in the bat stain intensely with Knitschitzky's hematoxylin as used in Baker's ('46) "pyridine extraction test" — pre-

sumptive evidence that they contain protein (fig. 2 B). The reaction of the granules in the other species to this test has not been recorded, but it is anticipated that they would be stained by it.

Different opinions have been held concerning the origin and significance of these acidophilic globules, not only in the bat, but in rodents as well. Branca ('23) interpreted those in the yolk sac of the bat as secretory granules, which in his opinion are "*. . . des signes indeniabiles d'une fonction glandulaire*" (p. 584). A similar view was held by earlier observers of the granules in rodents (Wislocki, Deane and Dempsey, '46, review the literature). Several facts speak against interpreting these globules in the bat as secretory granules: (a) Evidence of a secretory polarity is altogether lacking in the mesothelial cells, for the granules, especially when numerous, tend to lie at random in all parts of the cytoplasm and are not obviously related to a "Golgi zone." (b) Granules are not present either in the exocoelom or among the mesenchymal or vascular elements of the yolk-sac wall, so that they certainly are not extruded from the cells in a pre-formed state. (c) The low, non-fluctuating content of ribose nucleoproteins of the mesothelial cells suggests that no very active protein synthesis is being accomplished therein. (d) The folds and pleats which increase the area of the exocoelomic surface of the yolk sac (figs. 2 A, 23 and 32) are of the type more frequently associated with absorptive than secretory surfaces.

Recently investigators have proposed that the yolk-sac granules of the bat (Gerard, '28) and of rodents (Wislocki, Deane and Dempsey, '46) are directly absorbed materials. Gerard assumes that these materials are absorbed from the exocoelom in the bat, for he was able to demonstrate that the mesothelial cells are able to remove large quantities of trypan blue from this cavity, but he does not mention having observed the granules in the exocoelom. Wislocki, Deane and Dempsey conclude that the granules in the yolk-sac entoderm of rodents are absorbed from the cavity of the yolk sac and the uterine

lumen for they noted a correspondence between the tinctorial properties of the intracellular granules and the embryotrophic material in the adjoining cavities. They consider further that some of the granules (especially in the rat) resemble, and may be identical with, the fragments of extravasated maternal erythrocytes which are numerous in the contiguous embryotrophe, a possibility which in their opinion is supported by the fact that cytoplasmic iron, which is present in the entoderm cells, could be derived in part from such ingested fragments.

It is necessary to state emphatically that there is very little likelihood that the granules in the yolk sac of the bat are erythrocytic in origin, for blood cells were never observed in the exocoelomic or yolk-sac cavities, which are both permanently closed in the bat (fig. 1). Moreover, iron or hematogenous pigments do not occur in the mesothelial cells. Furthermore, in the exocoelom there are no acidophilic preformed materials resembling in any way the intracytoplasmic globules, so that the granules could not have been absorbed as such (the presence of fluid in the exocoelom may probably be assumed). On the contrary, the sequence in which granules appear in the cells, the smaller preceding the larger, strongly suggests that the granules arise *de novo* within the cytoplasm.

Another and heretofore unconsidered interpretation of the significance of the granules accords better with their general characteristics in the bat than the foregoing interpretations, and may have some bearing on similar granules in other species of animals. Gerard ('28) has clearly established by intra-vitam injection experiments that the mesothelial cells in the yolk sac of the bat are capable of absorbing substances from the exocoelom. Since the mesothelium of the yolk sac in the bat, the yolk-sac entoderm in rodents and the chorion in the pig have all been demonstrated to have an absorptive function, and since the granules in question occur in all, it is not illogical to consider that the granules may be associated in some way with the absorptive process. It does not follow, however, that they are the immediate materials absorbed, for it is evident in the yolk sac of the bat and in the chorion of the

pig that they are not, but they could be formed as extraneous products of the absorptive process.

In the bat, for example, progressive metabolic changes in the mesothelial cells of the yolk sac, possibly of a regressive nature, could presumably result in incomplete or improper utilization of absorbed materials and the appearance of non-utilizable proteinous by-products in the form of globular "degeneration bodies." The patchy distribution of cells containing the granules, the progressive increase of granules and granule-bearing cells as pregnancy advances, and the random distribution of the granules in the cytoplasm all attest the supervenient nature of the proteinous inclusions. If the granules were utilizable it might not be anticipated that they would progressively increase as they do in individual cells during pregnancy. If on the other hand the granules cannot be utilized, they are waste products, and if no mechanism exists for getting rid of them (and apparently none does in the bat since they are always confined to the epithelium) they will naturally accumulate as long as the metabolic processes responsible for their formation continue.

It is not without interest in connection with this view that glycogen likewise accumulates in the mesothelial cells, a phenomenon which suggests that the cells may be characterized by low respiratory metabolism (see p. 107). This in itself might be expected to have adverse effects on certain aspects of cellular physiology, including the absorptive function, and may be instrumental in the formation of the granules.

DISCUSSION

The more important features of the histochemistry of the placenta of the bat not yet completely dealt with will be discussed in reference to related observations in other mammals.

Significance of cytoplasmic basophilia

The cytoplasmic basophilia of a variety of cells has been attributed to their content of ribose nucleoprotein (Brachet, '40; Caspersson, '40; Gersh and Bodian, '43; Dempsey and Wislocki, '45; Dempsey and Wislocki, '46; Wislocki and

Wimsatt, '47; Dempsey and Singer, '46; Deane, '46; Dempsey, Bunting, Singer and Wislocki, '47; Noback and Montagna, '47). There is also evidence that the intensity of the basophilic staining is directly related to the quantity of nucleoprotein present (Brachet, '40; Greenstein, '44). It has been established, furthermore, that nucleoproteins are fundamentally involved in a number of important cellular activities, especially those concerned with the synthesis of protein for use within the cell, or as the proteinous component of a secretory product (Caspersson, '47; Hyden, '47; Bodian, '47). The works cited, and many others clearly indicate that fluctuations in the cellular content of nucleic acid may reflect important physiological changes in cells.

The distribution of nucleic acid in the placenta of the bat agrees in general with its distribution in the placentas of other mammals that have been investigated (man, pig, cat, rodents and shrews), but there are some significant points of difference.

Endometrium. In the bat and the other mammals that have been studied the endometrial components exhibit the most intense basophilia during progestation and the early stages of pregnancy. A gradual diminution of basophilia occurs thereafter. The periods of abundance of nucleic acid coincide with the periods of maximum growth and hypertrophy of the endometrial elements, and with the periods of maximum secretory activity by the uterine and glandular epithelia. Presumably, the increased nucleic acid of the epithelial cells is involved in the synthesis of the proteinous secretion which is visible in the uterine and glandular lumens at this time. The subsequent decrease in nucleic acid content is associated with a declining secretory activity of the epithelial cells. The connective tissue cells surrounding the implantation chamber in the bat and other mammals round up, enlarge and undergo decidual transformations. During their hypertrophy the cells are characterized by an intense basophilia, and the increased nucleic acid is presumably involved in the synthesis of protein necessary for cell growth. When maximum size has been attained basophilia,

and consequently the nucleic acid level, diminish, thereby indicating a decline in synthetic protein metabolism. Since all of the cells of the deciduae in the bat do not attain maximum hypertrophy simultaneously, an explanation is afforded for the variations in basophilic staining observed in different decidual zones (p. 68). Similarly, the occurrence of basophilia in the maternal endothelium during phases of proliferation and hypertrophy is accounted for (p. 69). It is significant, for example, that during the development of an endotheliochorial placenta the maternal endothelium undergoes an initial hypertrophy during which it is intensely basophilic. When maximum hypertrophy is attained the basophilia gradually diminishes and ultimately disappears (shrews, Wimsatt and Wislocki, '47, Wislocki and Wimsatt, '47; carnivores, Wislocki and Dempsey, '46a).

Trophoblast. The early appearance of an intense basophilia in the trophoblast is a characteristic of all of the mammalian species studied thus far, and is presumably correlated with the rapid growth and concomitant protein synthesis which characterizes this tissue. In those forms which develop a syncytial trophoblast the more intense basophilia appears to characterize this component of the trophoblast. In all species except the bat and rodents the basophilia of the syncytium rapidly declines after mid-gestation and all but disappears by term. In rodents it persists in the coarse (interlobular) syncytium, but disappears in the delicate syncytium of the labyrinth (Wislocki, Deane and Dempsey, '46). By contrast, the basophilia in the syncytial trophoblast of the bat not only does not disappear, but becomes more intense in the later stages of pregnancy. It does become restricted in distribution, however, being confined in late stages to the central and perinuclear zones of the syncytial tubules. It is quite likely that the intensification of staining results from an increased concentration of nucleic acid in the central zone to compensate for its loss in the peripheral zones, and does not represent an absolute increase in the syncytium as a whole. It may be mentioned parenthetically that the distribution of ribose nucleo-

protein in the syncytium during gestation closely approximates the distribution of alkaline nucleic acid phosphatase. This correspondence will be discussed in a later section (p. 117).

The distribution of nucleoprotein in the trophoblastic cells of the membranous chorion of the bat resembles its distribution in the corresponding cells of other mammals, except that it has not disappeared at term. The substance becomes segregated in the basal segments of the cells, but basophilia is still intense in near-term specimens, and is associated with a high cellular content of lipids (Wimsatt, '48).

Yolk sac. Pertinent histochemical descriptions of the yolk sac are available only for rodents (mouse, rat, guinea pig, rabbit; a study is made, and the literature is reviewed by Wislocki, Deane and Dempsey, '46), shrews (Wislocki and Wimsatt, '47) and the bat (Wimsatt, '48, and the present report). Basophilia is intense in all parts of the entodermal cells earlier in pregnancy in the bat, but becomes restricted to the apical, supranuclear cytoplasm in late pregnancy. In rodents the basophilia of the entodermal cells has been described as very intense throughout gestation. Changes in its localization during pregnancy are not recorded. In the mouse the basophilia appears to be concentrated basally ("infra-nuclearly"), whereas in the guinea pig the entire cytoplasm stains bluish. In shrews the entodermal cells are only moderately basophilic, the staining being most distinct basally, in the perinuclear region. Thus the apical localization of the basophilic material in the entodermal cells of the bat in late pregnancy is unique among the mammals studied thus far and the question arises as to whether this polarity bears any relation to the fact that the position of the Golgi apparatus is reversed in the entodermal cells of the bat where it is situated basally, rather than above the nucleus as in most other epithelia. The lipids of the entodermal cells are likewise most concentrated basally, in relation to the Golgi apparatus (Wimsatt, '48). The higher content of nucleoproteins in the entodermal than in the mesothelial cells of the yolk sac, to-

gether with other differences between these epithelia that have been discussed suggests that the entodermal cells may have a different, and perhaps greater, metabolic significance than the mesothelial cells.

Significance of placental glycogen

Factors favoring glycogen deposition in the placenta. Dempsey and Wislocki ('44) have hypothesized that glycogen is often deposited in tissues characterized by a low respiratory metabolism. They review numerous instances in both adult and fetal tissues where the accumulation of glycogen is associated with a low circulatory efficiency or actual ischemia, an observation also made by Creighton as long ago as 1896. They suggest further that glycogen deposition might be indicative of tissues in which anerobic metabolism prevails. Thus, in their words (p. 425), "... it is conceivable that glycogen would be deposited in tissues either because the metabolism, and therefore the rate of utilization of glycogen, was low, or because an intense anerobic metabolism demands a large accumulation of glycogen for the purpose of maintaining the fermentative process." They propose further (p. 425) that glycogen "may provide, through anerobic glycolysis, a source of energy for oxidations in those tissues which are deficient in their mechanisms for aerobic metabolism."

The distribution of glycogen in the placenta is consistent with the ischemia hypothesis. It may be shown that the accumulation of glycogen in both the maternal and fetal components of the normal placenta of various mammals (man, cat, dog, rodents, shrews) is associated with a reduced vascular efficiency (Wislocki and Dempsey, '45, '46a, '46b, '46c; Wislocki, Deane and Dempsey, '46; Wislocki and Wimsatt, '47). A similar conclusion is reached with respect to the excessive storage of glycogen in the chorionic trophoblast of two hydatidiform moles and in the villi of an eclamptic placenta (Wislocki and Dempsey, '46c).

The placenta of the bat may be added to the list of organs in which glycogen deposition is associated with a reduced

circulatory efficiency. The deposition of glycogen in the parietal decidua and glandular epithelium has been discussed (pp. 73 and 76). The much larger glycogen deposits in the cells of the decidua basalis arise in a region characterized by marked cellular proliferation and hypertrophy with accompanying histological manifestations of avascularity. In this zone capillaries become harder to find in the thickened endometrium, the afferent placental vessels are large and pass through the decidua without giving off branches, and only an inconsequential amount of blood is extravasated as the endometrium (and whatever capillaries it contains) is progressively eroded by the chorion. The accumulation of glycogen in the epithelial cells of the tubo-uterine junction (p. 74) is associated with the extensive sloughing and reorganization of this epithelium which occurs at the end of the progestational period.

In the visceral layer of the yolk sac glycogen is deposited in both the entodermal and mesothelial cells, but most abundantly in the latter. That the mesothelial cells may have a low respiratory metabolism is suggested by a number of factors: they have a very low nucleic acid content (pale basophilia), proteinous "degeneration bodies" progressively accumulate in their cytoplasm (see p. 99), and being components of folds which appear to be poorly vascularized they are probably less intimately related to the vitelline vessels than the entodermal cells (fig. 23). In addition to these factors it is pertinent that fetal blood is known to have a lower oxygen capacity and percentage saturation than maternal blood (Needham, '31 and '42), so that proximity to the vitelline capillaries would probably not preclude glycogenesis in any constituent of the yolk sac.

Vascular conditions in the discoidal placenta in early pregnancy may likewise favor glycogenesis in the cytotrophoblastic cells. Glycogen is most evident in the cells of the cytotrophoblastic cords nearer the deep (maternal) face of the placenta. The vascular patterns of the placental disc is such that richest maternal blood is discharged directly into the labyrinthine spaces just beneath the fetal surface of the

placenta whence it flows back through the deeper portions toward the basalis (Wimsatt, '45). Consequently, blood with a presumably lower oxygen tension occurs at the deeper levels of the placenta where glycogen deposits in the cytotrophoblastic cells are heaviest.

Inverse relation between glycogen and basophilia. Wislocki and Dempsey ('45) discuss the apparent inverse relation between the presence of glycogen and basophilic substance first reported by Bartelmez and Bensley ('32) in human endometrial glands, and extend the observation to other mammals. It is evident that a similar relation obtains in both the maternal and fetal placental constituents of the bat (e.g., cells of decidua basalis, uterine glands, cytotrophoblastic cords, mesothelium of yolk sac, etc.). Wislocki and Dempsey believe that the inverse relationship is incidental and does not indicate a chemical relationship between the two substances, a conclusion which is based on the assumption that the pentose sugar of the ribose nucleoprotein is not readily convertible into glycogen. The relation could be incidental also in the sense that a lowered nucleic acid level may conceivably accompany a lowered respiratory metabolism, which condition, according to the view discussed earlier, favors the storage of glycogen.

Significance of placental phosphatase

In summing up the numerous observations on phosphatase distribution in the bat placenta the following general features may be noted. Alkaline phosphatases are present throughout gestation and give more intense staining reactions than neutral or acid phosphatases. Alkaline and neutral phosphatases are more or less similarly distributed in the placenta and in general are found in the same locations within the cells. Acid phosphatase is almost entirely restricted to nuclei.

At alkaline pH with the different substrates the more intense reactions tend to be given with ribose nucleic acid, followed in descending order by Na- β -glycerophosphate, fructose 1-6 diphosphate, adenylic acid and lecithin. The reactions are variable at neutral pH, but are most constantly evoked with

the first three substrates. Nuclear reactions are more common with nucleic acid and adenylic acid, and are more evident at neutral and acid, than at alkaline ranges. Adenylic acid provided the most constant and widespread nuclear reactions at all pH levels. The nuclear reactions at acid pH are more marked in later pregnancy. Among the maternal tissues phosphatase activity declines in the uterine epithelium and in the cells of the decidua basalis during pregnancy, but increases in the mesometrial portion of the decidua parietalis and in the myometrium. One zone of the decidua, the layer of hypertrophied paraplacental cells, remains almost entirely free of phosphatase throughout gestation (it will be recalled that these same cells are rich in glycogen). The uterine endothelium is generally non-reactive. Among the fetal tissues characteristic phosphatase reactions are given by the syncytial trophoblast and the amniotic ectoderm. Phosphatase activity in the syncytium is most intense during the first half of gestation and falls off almost to extinction during the second half.² A decline of alkaline phosphatase activity in the syncytial trophoblast in later pregnancy characterizes only the bat and the guinea pig among the mammals thus far studied, for in the cat (Wislocki and Dempsey, '46a), mouse, rat (Wislocki, Deane and Dempsey, '46) and man (Dempsey and Wislocki, '44; Wislocki and Dempsey, '46c) there is a progressive increase in enzymatic activity as pregnancy advances. These

² Stafford and Atkinson ('48) have studied quantitatively the effects of alcohol and acetone fixation, paraffin embedding and sectioning on alkaline and acid phosphatase activity in a variety of tissues. They conclude that acid phosphatase is inactivated by both alcohol and acetone to a far greater extent than alkaline phosphatase, that alcohol preserves a greater amount of alkaline phosphatase activity than acetone, and that the enzyme activity remaining in paraffin-embedded tissue sections is approximately 5% in the case of acid phosphatase and 20-30% in the case of alkaline phosphatase. Such factors as these may explain not only the apparent deficiency in acid phosphatase in the placenta, but may account for the weak reactions of the syncytium in late pregnancy. Since all of the materials in the present study were similarly prepared, however, and would presumably be influenced to the same degree by inhibiting factors, it is believed that the described fluctuations in phosphatase activity in all probability do reflect actual changes in enzymatic activity during gestation.

observations suggest that the labyrinthine placenta of the bat, like that of the guinea pig (Hard, '46), undergoes wide fluctuations in activity during its existence, fluctuations that are probably related to the physiological differentiation of other fetal tissues. The amnion gives the most intense reactions of any fetal tissue and it alone possesses phosphatases that will attack nearly all of the substrates used in each of the three pH levels. Enzymatic activity is most pronounced, however, at alkaline pH. With the exception of the nuclear enzymes, phosphatases are usually found in the trophoblastic cells of the membranous chorion only opposite endometrial zones containing the enzymes, and give every manifestation of having been absorbed by the trophoblast. Although the series of yolk sacs available for the study of phosphatases was incomplete, the observations made suggest that greater enzymatic activity is encountered after the middle of gestation than before, and that activity might decline somewhat in the final stages. Hard ('46) describes a similar decline in enzyme activity in the yolk sac of the guinea pig.

Specificity of the placental phosphatases. The diversity of the results obtained with the various substrates at different levels of pH raises the question as to whether a single enzyme capable of attacking a variety of substrates is responsible, or whether a number of enzymes are present which act more or less specifically on the different substrates. The question of phosphatase specificity is reviewed in several recent papers (Dempsey and Wislocki, '47; Zorzoli and Stowell, '47; Lison, '48), and it is concluded that the available biochemical evidence is insufficient for determining the degree of specificity of phosphatases toward different substrates. Presumptive histochemical evidence for the presence of several distinct phosphatases in the placenta has been provided, however, by Dempsey and Wislocki ('47), who observed different morphological localizations of enzyme activity when different substrates were used.

Differences in the localization of enzyme activity in response to different substrates were also observed in the present

study. The differing reactions of the nuclei when various substrates are used provide an excellent example, but especially striking are the differential reactions obtained in the syncytial trophoblast when glycerophosphate and ribose nucleic acid are used as substrates. It will be recalled that the outer portion of the syncytium adjoining the lumen of the tubule shows an intense reaction with glycerophosphate, the remainder of the syncytium a much milder one. With ribose nucleic acid the situation is reversed, the outer zone being essentially negative, the remaining syncytium reacting more strongly than it does with glycerophosphate. If differential results are to be interpreted as the work of a single phosphatase, then it must be assumed in order to explain the differential staining that the enzyme acts more efficiently on one substrate than the other. Since the most intense reaction of all is given in the outer zone with glycerophosphate it might be further assumed that glycerophosphate is the preferred substrate, and that the differing intensities of the reactions to glycerophosphate in the outer and inner zones reflect regional differences in the concentration of the enzyme. But if glycerophosphate were the preferred substrate, how could one account for the heavier reaction with nucleic acid than with glycerophosphate in the inner zone? And since the hypothetical single enzyme is capable of reacting with nucleic acid in the inner zone why does it not do so in the outer zone of the syncytium which displays intense enzyme activity with other substrates? It would therefore seem that an enzyme capable of splitting glycerophosphate is present in all of the syncytium of the bat, but in greatest concentration during the first half of pregnancy in a narrow outer zone, whereas an enzyme capable of splitting ribose nucleic acid is confined to the inner zone of the syncytium, and does not occur in the outer zone. In short, the type of inverse localization of enzymatic activity that is shown with glycerophosphate and yeast nucleic acid in the syncytium does not accord with the concept of a single phosphatase, but harmonizes better with the assumed presence of specific enzymes (at least two in the described in-

stance). Only when localization of enzymatic activity with two substrates is the same (as with glycerophosphate and fructose diphosphate in the outer syncytium) can the concept of an hypothetical single enzyme be supported on morphological grounds.

The role of placental phosphatases. The possible role of phosphatases in a number of important metabolic processes has been outlined in biochemical and physiological studies. For example, the enzymes are phosphorylating agents in the conversion of glycogen to glucose (Cori, '41), and there is evidence that they probably are involved in the transport of materials across cell membranes, as in the phosphorylation of lipids for absorption by the intestinal epithelium (Bloor, '43; Sumner and Somers, '43). It is only logical to assume that similar functions may be performed by the phosphatases of the placenta where an active carbohydrate and lipid metabolism is known to prevail (Needham, '31; '42). Hard ('46) observed correlated fluctuations in alkaline phosphatase activity on the one hand and concentrations of glycogen and fat on the other in the placenta of the guinea pig, and concluded that the enzymatic activity is probably associated with phospholipid and sugar metabolism. A similar conclusion is reached with respect to glycogen deposition in the placenta of the sow, cat, rodents and man by Wislocki and Dempsey ('45) who find that a region containing phosphatase invariably intervenes between the maternal blood stream and the locality where glycogen is deposited. They suggest that the dephosphorylation of "hexosephosphate" by alkaline phosphatase is probably a necessary antecedent to glycogen formation — an hypothesis which has compelling biochemical support.

In the placenta of the bat similar relations between phosphatase, glycogen and lipids in general prevail. The intense phosphatase activity of the deciduae is associated with prominent intracellular deposits of lipids (Wimsatt, '48) and glycogen. The outstanding exception is provided by the cells of the paraplacental decidua which are loaded with glycogen, but in which phosphatases are only active in the nuclei. This

relatively narrow decidual zone is bounded on the one side, however, by the phosphatase-rich placental labyrinth and on the other by the deeper decidua basalis, which also contains abundant phosphatase during the earlier periods of pregnancy. In the mesothelial cells of the yolk sac the presence of glycogen is associated, at least in the latter half of pregnancy, with irregular reactions for phosphatase in the vitelline endothelium (p. 89). The phosphatases irregularly encountered at the apices of the mesothelial cells may have been absorbed from the exocoelom. It was previously suggested on histochemical grounds that the labyrinthine placenta of the bat probably plays an active role in the lipid metabolism of the fetus (Wimsatt, '48). The presence of phosphatases in the placental barrier may provide a mechanism whereby this is accomplished, for it is conceivable that phosphorylation of circulating fats by the phosphatases of the syncytium may occur and thereby facilitate lipid absorption by the trophoblast. It is also of interest in this connection that the trophoblastic cells of the subplacental membranous chorion contain during the later stages of pregnancy heavy deposits of neutral fat, phospholipid and cholesterids (Wimsatt, '48). Although cytoplasmic phosphatase activity in these cells is irregular, the adjoining decidua from which these cells actively absorb materials contains an abundance of enzymes, and irregular reactions have also been observed in the endothelium of the allantoic vessels underlying the chorionic epithelium. It is therefore possible that phosphatases may be involved in the lipid metabolism and transport in this portion of the placental barrier.

In a number of histochemical studies of the placenta Wislocki and Dempsey ('45, '46a, and '46b), Wislocki, Deane and Dempsey ('46), Wislocki and Wimsatt ('47) have demonstrated that a layer of (alkaline) phosphatase is consistently interposed between the maternal and fetal circulations, and most often at the actual boundary between maternal and fetal tissues. In hemochorial placentas the enzyme is ordinarily present in the outermost trophoblastic layer, and it occupies

a similar position in the two endotheliochorial placentas thus far investigated (cat and shrew). This situation probably finds an explanation in the importance of the phosphorylation mechanism to a variety of metabolic processes which must be carried on at the placental barrier, and accords with the interpretation of the barrier as a "selective" membrane. It is apparent from the results of the present study that the hemochorial placenta of the bat shows a similar disposition of phosphatase in the trophoblastic barrier, and the bat therefore constitutes no exception to the general rule.

The question of an inverse relationship between alkaline phosphatase and basophilia. Wislocki and his collaborators in studies of placental histochemistry have indicated that in the placental barrier of a variety of mammals (rodents, cat, shrews, man) alkaline phosphatase and basophilia (ribose nucleoproteins) bear an inverse relationship to one another in the sense that basophilia declines as pregnancy advances, whereas alkaline phosphatase increases, and the implication is that the two are causally related. Dempsey and Wislocki ('45) in describing the human placenta have even stated that alkaline glycerophosphatase and basophilia appear to be "incompatible." Thus, in the syncytial trophoblast of the human chorionic villus basophilia recedes to deeper levels as the more superficially situated alkaline phosphatase progressively encroaches upon its territory. (Hard, '46, has shown that in the placenta of the guinea pig alkaline glycerophosphatase as well as basophilia declines during the last quarter of gestation, but this in no sense invalidates the inverse relationship which is manifest during the earlier stages.) In explanation of the phenomenon Dempsey and Wislocki ('45) suggest that nucleic acids as well as their constituent nucleotides may be regarded as phosphoric esters, and hence possible substrates for phosphatase. They suggest the possibility that enzymatic hydrolysis of ribonucleic acid by the increasing alkaline phosphatase in the syncytium produces the decrease in basophilia as pregnancy advances.

The relationship of the alkaline phosphatase and basophilia in the syncytial trophoblast of the bat constitutes an exception to the situation prevailing in other mammals. In the syncytium of this species alkaline glycerophosphatase is more abundant during the first half of pregnancy, when basophilia is most widespread. Later, phosphatase activity markedly declines, whereas basophilia persists. Furthermore, whereas the picture in early pregnancy of a superficial, narrow, non-basophilic zone intensely positive for phosphatase adjoining a deeper, basophilic zone with reduced enzymatic activity is suggestive of the inverse relation observed in other mammals, the fact remains that the retreat of basophilia to the deeper levels of the syncytium of the bat is not accompanied by a corresponding advance of phosphatase. Indeed, the outer zone of intense enzymatic activity never widens, and shortly after mid-gestation disappears altogether. With the exception of the narrow outer zone, where alkaline glycerophosphatase occurs in the absence of nucleoproteins the two substances co-exist in the same parts of the syncytial cytoplasm as long as phosphatase is present, so that in the syncytium of the bat these substances cannot be said to be "incompatible."

The view expressed by Dempsey and Wislocki ('45) that the nucleic acid of the syncytium in man might be hydrolyzed by "glycerophosphatase" and thereby account for the inverse relationship of these two substances is based entirely on the observed morphological distribution of ribose nucleic acid and phosphatase and can only be substantiated by quantitative biochemical methods. Whether or not the view is ultimately proved valid, the relationship of the two substances in the syncytium of the bat provides no morphological support for it. Furthermore, in light of the results obtained in the bat and in man when ribose nucleic acid rather than glycerophosphate is used as substrate, one might question whether the "glycerophosphatase" is actually capable of attacking nucleic acid as suggested by Dempsey and Wislocki ('45). In the bat, it will be recalled, the intense phosphatase activity observed in the inner zone of the syncytium with glycerophosphate is

absent with nucleic acid, presumably indicating the inability of the enzyme at this location to hydrolyze the latter substrate. That an enzyme which is capable of splitting ribose nucleic acid exists, however, is attested by the positive reaction which occurs in the rest of the syncytium. It is perhaps more than coincidence that throughout pregnancy the distribution of nucleic acid phosphatase in the syncytium of the bat corresponds almost exactly with the distribution of ribose nucleoprotein (compare figs. 6 and 36), an observation which provides some (but not conclusive) support for the presumed presence in the syncytium of a specific nucleic acid-splitting enzyme.

Furthermore, in the human placental syncytium the distribution of nucleic acid phosphatase does not coincide with that of glycerophosphatase (Dempsey and Wislocki, '47), and does not, therefore, stand in inverse relation to basophilia in the morphological sense. The nucleic acid enzyme of the human syncytial trophoblast at term, unlike the nucleic acid enzyme of the bat, and unlike the placental glycerophosphatase of man, is uniformly distributed in all of the syncytial cytoplasm, so that an enzyme known to be able to split nucleic acid occurs alike in regions that contain and lack nucleoprotein.

In the placental syncytium of the cat and rodents on the other hand, nucleic acid phosphatase appears to have the same distribution as glycerophosphatase (Dempsey and Wislocki, '47), and the distribution of the enzymes in these placentas therefore provides no information toward the settlement of the question of the substrate specificities of the placental phosphatases.

In some other placental tissues of the bat on the other hand, an apparent inverse relationship between alkaline phosphatase and basophilia is observed. It may be recalled that the cellular trophoblast of the implanting blastocyst is intensely basophilic, but is negative for phosphatase. Shortly after implantation the "basal cytotrophoblast" of the placental disc is phosphatase-negative, but intensely basophilic, and the cytotrophoblastic cells of the primary villi reveal a declining

basophilia, but do show some phosphatase activity. The reconstituted uterine epithelial cells at the mesometrial pole of the gestation chamber have a reduced basophilia apically and reveal apical phosphatase activity. On the other hand, the uterine epithelial cells and those of the glands during progestation are at the same time intensely basophilic and show high phosphatase activity, although in the glandular cells phosphatase is most abundant in the apical cytoplasm where basophilia is least conspicuous. In most of the above instances, however, the distribution of nucleoprotein and phosphatase in relation to one another is not precise enough to indicate that the apparent inverse relation is anything more than incidental.

Significance of placental iron

All of the iron required by the fetus must be provided by the maternal organism. Three maternal sources of iron have been recognized in the placentas of diverse mammals, viz., the uterine glands, which secrete iron during pregnancy in some species (shrews, pig, rat, guinea pig and man) but not in others (cat, dog, bat); the extravasated maternal erythrocytes, which are destroyed in small numbers in the uterine lumen (rodents), or in large numbers in the specialized placental "hematomata" of a variety of mammals (insectivores, carnivores, sheep, cow, etc.); and the maternal blood plasma, from which iron may be extracted directly by the trophoblast (man, guinea pig, bat, etc.). Histochemical evidence suggests that these sources do not have equal significance in various species. In at least one form, the pig, the only available iron appears to be that secreted by the uterine glands, and to a lesser extent by the surface epithelium. The secreted iron is absorbed through all parts of the chorion, but principally by the areolae, which lie opposite the openings of the glands (Wislocki and Dempsey, '46b). A similar arrangement might logically be expected in any other species which possesses an epitheliochorial placenta and maintains an intact endometrium throughout pregnancy. On the other hand iron is also secreted

by the uterine glands in some of those mammals in which the endometrium is eroded to a greater or lesser degree during pregnancy (e.g., man, rat, guinea pig, shrews), but the histochemical picture in these species strongly suggests that secreted iron is of only subsidiary importance, the major proportion of the fetal iron being derived from one or both of the other sources mentioned. Finally there is a group of mammals (cat, dog, Wislocki and Dempsey, '46a; and bat) in which endometrial erosion takes place, but in which iron is not demonstrable histochemically in the uterine glands or surface epithelium and presumably is not secreted by the endometrium.

The relative significance of extravasated maternal erythrocytes and of the maternal blood plasma as sources of fetal iron undoubtedly depends upon the degree and nature of endometrial erosion and upon the nature of the placental barrier which is ultimately established. As far as can be determined histochemically extravasated maternal blood appears to be the principal source of fetal iron in species where the erosion process is incomplete in the sense that endometrial connective tissue and/or endothelium is left to form a part of the definitive placental barrier, that is, in species possessing the so-called "syndesmochorial" and "endotheliochorial" types of placenta. In the sheep and cow which have syndesmochorial placentas, for example, true hematomata develop about the bases of the fetal cotyledons, the degenerating maternal corpuscles being destroyed in enormous quantities by the chorionic cells between the roots of the villi (unpublished personal observations; see also Assheton, '06). The marginal hematomata of the carnivore placenta, which is apparently most often of the endotheliochorial labyrinthine type, are too well known for further comment. Their importance to the iron metabolism of the fetus in the cat and dog has most recently been discussed by Wislocki and Dempsey ('46a). A somewhat less conspicuous annular hematoma likewise characterizes the endotheliochorial placenta of shrews (Wimsatt and Wislocki, '47; Wislocki and Wimsatt, '47). The absence of any histochemically demonstrable iron in the placental labyrinth of

the endotheliochorial placentas in which a specific search has been made for it (cat, dog, shrews), especially in view of the positive reactions that are evoked in hemochorial placentas, provides support for the hypothesis that the hematomata represent the principal source of fetal iron, for it suggests that iron in the circulating maternal plasma is not transmitted across the endotheliochorial barrier in appreciable amounts.⁶

On the other hand, some histochemically detectable iron has been observed in the placental barrier of all hemochorial placentas examined for it (e.g., man, Dempsey and Wislocki, '44; rodents, Chipman, '02; Wislocki, Deane and Dempsey, '46; bat, present study). Iron is abundant in the syncytial trophoblast of human chorionic villi in early pregnancy, and is irregularly present in the parasyncytial stroma during the second and third quarters of gestation (Wislocki and Dempsey, '46c). It is concluded that most of the iron has been absorbed directly from the maternal blood and is being passed across the placental barrier to the fetal circulation. Only traces of iron were found in the hemochorial labyrinthine placentas of various rodents (mouse, rat, guinea pig, hamster) by Wislocki, Deane and Dempsey, ('46), the Turnbull blue reactions in the placental syncytium being characterized simply as "faint and diffuse." On the other hand Chipman ('02) found iron in the mesenchymal stroma of the labyrinthine placenta of the rabbit beginning on the 14th day, and by the 18th day traces were also visible in portions of the syncytium. That in the syncytium later disappeared, but the stromal iron persisted until term. Histochemically detectable iron is relatively more abundant in the yolk-sac endoderm of rodents than in the labyrinthine placenta, and represents absorbed iron that originates both from the uterine glands and from extravasated maternal erythrocytes (Wislocki, Deane and Dempsey, '46). From the histochemical picture alone the

⁶The ability of the endotheliochorial barrier to transmit iron could and should be tested quantitatively, however, by tracing the course of radioactive iron injected into the maternal blood stream during pregnancy as was done by Flexner, Vosburgh and Cowie ('48) in the guinea pig.

conclusion might be drawn that the endometrium and extravasated erythrocytes constitute the principal sources of fetal iron in rodents. This appears highly unlikely, for as a result of recent experiments conducted by Flexner, Vosburgh and Cowie ('48) it has been shown that the absorption of iron from the maternal plasma in the labyrinthine placenta of the guinea pig exceeds by a considerable margin the daily fetal requirement at the three ages tested.⁷ The authors conclude that plasma iron is sufficient to meet the fetal need for iron during pregnancy in the guinea pig.

Histochemically demonstrable iron is present in the hemochorial labyrinthine placenta of the bat, but this species differs from the various species of insectivores, rodents, carnivores and ungulates mentioned above in that it lacks the well-defined subsidiary sources of iron which characterize the placentas of many of these animals. There is no endometrial secretion of iron that can be absorbed by the trophoblast, and erosion of the endometrium is accomplished in a way, and at a rate which permits only a very irregular and negligible extravasation of maternal blood, and consequently iron is rarely demonstrable in the membranous chorion. The conclusion therefore appears justified that practically all of the iron required by the fetus in the bat is absorbed directly from the maternal blood by the syneytial trophoblast of the placental labyrinth.

The distribution of iron in the labyrinthine placenta of the bat closely resembles its distribution in the hemochorial placentas of other mammals studied (e.g., man, rodents) in regard to the following particulars: (a) it is principally concentrated in the stroma of the deeper half of the placental disc adjoining the trophoblast, beneath which in many places it appears to form a narrow but continuous layer, (b) instead

⁷ At day 40 the ratio of the fetal per diem requirement to the amount supplied is 60:100 μ g; at day 55 the ratio is 170:200 μ g; and near term the ratio is 250:370 μ g. The authors also conclude that at day 40 a maximum of 20 μ g of iron are derived daily from maternal red cells, and that consequently at this age the fetus is largely dependent upon iron from maternal plasma.

of being uniformly distributed in the stroma of the regions where it occurs the iron shows a patchy localization, and there is a general tendency for the histochemically demonstrable iron to decline in amount in the terminal phases of gestation. In the human placenta the stromal iron is associated with deposits of calcium and phosphatase, but this association is believed to be incidental by Wislocki and Dempsey ('46c). No such association has been observed in the placenta of the bat.

The suggestion has been made that the patchy distribution of stromal iron in the human placenta, and the tendency of these deposits and the associated phosphatase and calcium to be localized in the intermediate or terminal portions of the villi may depend upon fetal circulatory deficiencies in the regions where the substances occur, although the existence of such deficiencies has not yet been demonstrated (Wislocki and Dempsey, '46c, briefly discuss the point and review the pertinent literature). This suggestion would seem to imply that in regions where the fetal circulation is inefficient iron is absorbed from the maternal blood by the trophoblast at a faster rate than it can be removed by the fetal circulation, with the result that it accumulates in the fetal stroma.

Such a view would certainly appear to have more in its favor in the present state of knowledge than any teleological explanation, but an alternative view also appears to be admissible. It is suggested that the character of the maternal circulation in labyrinthine placentas (and possibly also in the villous placenta of man) may provide the trophoblast with greater opportunities to absorb iron in the deeper parts of the labyrinth than in the more superficial portions, and that the greater quantities observed may therefore reflect greater absorptive activity (for iron) in the deeper zone. This view as it applies to the bat rests upon two morphological characteristics of the placenta: (a) the direction of flow of maternal blood through the placental tubules is from the superficial to the deep surface of the disc (as it apparently is in all labyrinthine placentas thus far investigated, Mossman and Weisfeldt,

'39), and (b) the lumina of the trophoblastic tubules are markedly wider in the central portions of the deeper zone than in the more superficial zones of the placenta and consequently a slowing of the maternal circulation in the deeper zone may probably be assumed. It is conceivable that the slower circulation of the blood and its lowered nutrient value and oxygen tension in the deeper portion of the placenta could facilitate the absorption of iron from the plasma, and thereby account for its greater abundance in this region.

SUMMARY AND CONCLUSIONS

The distribution and significance of the following substances in the placenta of the bat are discussed: cytoplasmic basophilic substances (ribose nucleoproteins), glycogen, phosphatases, lipase, argyrophilic materials (Bodian protargol method), iron, metachromatic substances, and acidophilic inclusions in the free segment of the yolk sac.

Maximum basophilic staining in the individual endometrial tissues coincides with periods of maximum growth or functional activity. In the epithelium of the glands and the endothelium of the maternal vessels at the implantation site this occurs during progestation and the early post-implantation period. Among the connective tissue cells of the deciduae, those of the basalis display maximum basophilia during the first two-thirds of gestation, those of the parietalis during the last half. Once hypertrophy has commenced in the decidual cells an inverse relation between cell size and the degree of basophilic staining becomes established. Among the fetal tissues of the placenta cytoplasmic basophilia is nearly universal during the early periods of the establishment of the placental labyrinth and the differentiation of the other fetal membranes. It persists throughout gestation in all of the major elements, but becomes more restricted in distribution. An inverse relationship, that has been described in other species between basophilia and alkaline phosphatase in the placental syncytium is not apparent in the bat. An inverse relationship between basophilic substance and glycogen does occur in the placental

tissues, but it is improbable that the two substances have a chemical relationship.

Glycogen accumulates in enormous quantities at two sites, one maternal and the other fetal, namely, in the paraplacental decidual layer which intervenes between the placental disc and the deeper decidua basalis, and in the columnar mesothelial cells covering the exocoelomic surface of the yolk sac. In the latter it persists until term. It occurs also in lesser quantities in the other decidual cells, uterine glandular epithelium, amniotic ectoderm, membranous chorion (trophoblastic cells), and cytotrophoblast of the placental disc. It was never observed in the syncytial trophoblast of the labyrinth. The endometrial glycogen is absorbed by the membranous chorion as the endometrium is eroded. The distribution of glycogen in the placental tissues of the bat is such as to lend support to the hypothesis of Dempsey and Wislocki ('44) that glycogen deposition tends to occur in tissues characterized by a low respiratory metabolism.

Phosphatase activity was investigated with a number of substrates (Na- β -glycerophosphate, ribose nucleic acid, fructose 1-6 diphosphate, adenylic acid, lecithin) at three pH levels, 9.4, 7.0 and 4.7. Alkaline phosphatases are most consistently present in the placenta throughout pregnancy. Neutral phosphatases are similarly distributed but have a much lower activity. Acid phosphatases are confined almost exclusively to nuclei and increase during pregnancy. In general, nucleic acid gave the most intense reactions, followed by Na- β -glycerophosphate and fructose diphosphate. Nuclear reactions were more often elicited with nucleic acid than with the latter two substrates, but not as often as with adenylic acid. Phosphatase activity is high in the uterine glands, but declines after implantation. It is high also in the decidua basalis in early pregnancy, and in the decidua parietalis mesometrially in late stages. The myometrial reaction is intense during most of gestation. Phosphatase activity in the placental syncytium is highest during the first half of gestation, and declines nearly to extinction during the second half.

Thus a layer of phosphatase intervenes between the maternal and fetal circulations in the bat just as it does in the placentas of other mammals that have been investigated. Phosphatase is practically absent in the trophoblastic cells of the membranous chorion at all stages, and is present in the wall of the yolk sac only irregularly during the second half of pregnancy. The most intense phosphatase activity at all stages of pregnancy is given by the ectodermal cells of the amnion. Differences in the morphological localization of phosphatase activity with different substrates are described, and are interpreted to indicate that several phosphatases differing in their specificity toward various substrates are present in the placenta. In the placenta of the bat as in other mammals a layer of phosphatase always intervenes between the maternal (or fetal) blood stream and the locality where glycogen is deposited. The possible significance of placental phosphatase in carbohydrate and lipid metabolism is discussed.

Lipase could not be demonstrated in the placenta of the bat at any of the three stages of gestation at which it was sought.

Argyrophilic granules stained by the Bodian protargol method are confined to the zone of necrosis between the paraplacental decidua and the deeper basalis, and to occasional granules in the subplacental chorionic cells. The granules are not acid-soluble and presumably, therefore, are not deposits of calcium salts. It is suggested that they might represent impregnated pigment granules.

Iron has a very restricted distribution in the placenta. The most characteristic reactions occur patchily in the mesenchymal stroma of the deeper portions of the placental labyrinth immediately beneath the syncytial trophoblast (Turnbull blue and Prussian blue). A faintly positive Macallum reaction (for "organic" iron) is given in the syncytial trophoblast. It is concluded that the fetal iron in the bat is derived almost entirely from the maternal blood in the placental labyrinth. The relative importance of various sources of fetal iron is discussed from the standpoint of the comparative histology of the placenta and correlated histochemical observations.

Metachromasia was observed only in the granules of mast cells, which are numerous in the myometrium, and in the mucus of the cervical glands. The cervical mucus was also positive to the Bauer-Feulgen reactions, but the mast granules appeared to be negative to this test. These staining reactions were not altered in sections treated with hyaluronidase.

The distribution and certain solubility and staining characteristics of globular proteinous inclusions in the mesothelial cells covering the exocoelomic surface of the yolk sac are described. The inclusions have been variously interpreted in the past as secretion granules or absorbed substances. The heretofore unconsidered possibility that the granules may represent non-utilizable waste products formed superveniently to the absorptive process is discussed.

LITERATURE CITED

- ASPLUND, J., U. BORELL AND H. HOLMGREN 1940 In der Uteruswand während der Gravidität auftretende metachromatisch granuliert Zellverbände und ihre Stellung zur "Glandula myometrialis." *Zeitsch. f. mikr.-anat. Forsch.*, 48: 478-528.
- ASSHETON, R. 1906 VI. The morphology of the ungulate placenta, particularly the development of that organ in the sheep, and notes upon the placenta of the elephant and Hyrax. *Phil. Trans. Roy. Soc. Lond., Ser. B*, 198: 143-220.
- BAKER, J. R. 1946 The histochemical recognition of lipine. *Quart. J. Micr. Sci.*, 87: 441-470.
- BENSLEY, R. R., AND S. H. BENSLEY 1938 *Handbook of Histological and Cytological Technique*. Univ. of Chicago Press.
- BLOOR, W. R. 1943 *Biochemistry of the Fatty Acids*. A. C. S. Monograph Ser. Reinhold Publishing Corp., New York.
- BODIAN, D. 1947 Nucleic acid in nerve-cell regeneration. *Soc. Exp. Biol. Symposia*, 1, Nucleic Acid: 163-178. Cambridge Univ. Press.
- BRACHET, J. 1940 La detection histochemique des acides pentosenucleiques. *C. R. Soc. Biol.*, 133: 88-90.
- BRANCA, A. 1923 Recherches sur la vésicule ombilicale. II. La vésicule ombilicale des cheiroptères. *Arch. d. Biol.*, 33: 517-604.
- CASPERSSON, T. 1940 Methods for the determination of the absorption spectra of cell structures. *J. Roy. Micr. Soc.*, 60: 8-25.
- 1947 The relations between nucleic acid and protein synthesis. *Soc. Exp. Biol. Symposia*, 1, Nucleic Acid: 127-151. Cambridge Univ. Press.

- CHIPMAN, W. W. 1902 Observations on the placenta of the rabbit, with special reference to the presence of glycogen, fat, and iron. *Studies from the Royal Victoria Hosp., Montreal*, 1: 225-485.
- CORI, C. F. 1941 Phosphorylation of glycogen and glucose. *Biol. Symposia*, 5: 131-140. J. Cattell Press.
- CREIGHTON, C. 1896 *Microscopic Researches on the Formative Property of Glycogen*. Black, London.
- DAWSON, A. B., AND J. BARNETT 1944 Bodian's protargol method applied to other than neurological preparations. *Stain Tech.*, 19: 115-118.
- DAWSON, A. B., AND B. A. KOSTERS 1944 Preimplantation changes in the uterine mucosa of the cat. *Am. J. Anat.*, 75: 1-37.
- DEANE, H. W. 1946 The basophilic bodies in hepatic cells. *Am. J. Anat.*, 78: 227-244.
- DEMPSEY, E. W., H. BUNTING, M. SINGER AND G. B. WISLOCKI 1947 The dye-binding capacity and other chemo-histological properties of mammalian mucopolysaccharides. *Anat. Rec.*, 98: 417-430.
- DEMPSEY, E. W., AND M. SINGER 1946 Observations on the chemical cytology of the thyroid gland at different functional states. *Endocrinol.*, 38: 270-295.
- DEMPSEY, E. W., AND G. B. WISLOCKI 1944 Observations on some histochemical reactions in the human placenta, with special reference to the significance of the lipoids, glycogen and iron. *Endocrinol.*, 35: 409-429.
- 1945 Histochemical reactions associated with basophilia and acidophilia in the placenta and pituitary gland. *Am. J. Anat.*, 76: 277-301.
- 1946 Histochemical contributions to physiology. *Physiol. Rev.*, 26: 1-27.
- 1947 Further observations on the distribution of phosphatases in mammalian placentas. *Am. J. Anat.*, 80: 1-33.
- ERNST, M. 1926 Über Untergang von Zellen während der normalen Entwicklung bei Wirbeltieren. *Zeitsch. f. Anat. u. Entwicklungs.*, 79: 228-262.
- FLEXNER, L. B., G. J. VOSBURGH AND D. B. COWIE 1948 Sources of fetal iron in the guinea pig as determined with radioactive iron. *Anat. Rec.*, 100: 661. (Abstract.)
- GÉRARD, P. 1928 Recherches histophysiologiques sur les annexes fœtales des cheiroptères (*Vesperugo noctula* Schreb.). *Arch. d. Biol.*, 38: 327-354.
- GERSH, I., AND D. BODIAN 1943 Histochemical analysis of changes in Rhesus motoneurons after root section. *Biol. Symposia*, 10: 163-184.
- GOMORI, G. 1941 The distribution of phosphatase in normal organs and tissues. *J. Cell. and Comp. Physiol.*, 17: 71-83.
- 1945 The microchemical demonstration of sites of lipase activity. *Proc. Soc. Exp. Biol. and Med.*, 58: 362-364.
- GREENSTEIN, J. P. 1944 Nucleoproteins. *Advances in protein chemistry*, 1: 210-281. Academic Press, Inc., New York.
- HARD, W. L. 1946 A histochemical and quantitative study of phosphatase in the placenta and fetal membranes of the guinea pig. *Am. J. Anat.*, 78: 47-77.

- HOLMGREN, H. 1940 Studien über Verbreitung und Bedeutung der chromotropen Substanz. *Zeitsch. f. mikr.-anat. Forsch.*, 47: 489-521.
- HYDÉN, H. 1947 Protein and nucleotide metabolism in the nerve cell under different functional conditions. *Soc. Exp. Biol. Symposia*, 1: Nucleic Acid: 152-162. Cambridge Univ. Press.
- KUNITZ, M. 1940 Crystalline ribonuclease. *J. Gen. Physiol.*, 24: 15-32.
- LISON, L. 1936 *Histochimie Animale*. Gauthier-Villars, Paris.
- 1948 La recherche histochimique des phosphatases. *Étude critique*. *Bull. d'histol. appl. et de Tech. Microscop.*, 25: 23-41.
- MITCHELL, A. J., AND G. B. WISLOCKI 1944 Selective staining of glycogen by ammoniacal silver nitrate: a new method. *Anat. Rec.*, 90: 261-266.
- MOSSMAN, H. W., AND L. A. WEISFELDT 1939 The fetal membranes of a primitive rodent, the thirteen-striped ground squirrel. *Am. J. Anat.*, 64: 59-100.
- NEEDHAM, J. 1931 *Chemical Embryology*. III. Cambridge Univ. Press, London.
- 1942 *Biochemistry and Morphogenesis*. Cambridge Univ. Press, London.
- NOBACK, C. R., AND W. MONTAGNA 1947 Histochemical studies of the basophilia, lipase and phosphatases in the mammalian pancreas and salivary glands. *Am. J. Anat.*, 51: 343-368.
- PALAY, S. L. 1945 Neurosecretion. VII. The preoptico-hypophysial pathway in fishes. *J. Comp. Neurol.*, 82: 129-143.
- REEDER, E. 1939 Cytology of the reproductive tract of the female bat, *Myotis lucifugus*. *J. Morph.*, 64: 431-453.
- SCOTT, G. 1933 The localization of mineral salts in cells of some mammalian tissues by microincineration. *Am. J. Anat.*, 53: 243-287.
- SCHARRER, E. A., AND B. SCHARRER 1945 Neurosecretion. *Physiol. Rev.*, 25: 171-181.
- STAFFORD, R. O., AND W. B. ATKINSON 1948 Effect of acetone and alcohol fixation and paraffin embedding on activity of acid and alkaline phosphatases in rat tissues. *Science*, 107: 279-281.
- SUMNER, J. B., AND G. F. SOMERS 1943 *Chemistry and Methods of Enzymes*. Academic Press, Inc., N. Y.
- WACHSTEIN, M. 1945 Influence of experimental kidney damage on histochemically demonstrable lipase activity in the rat. Comparison with alkaline phosphatase activity. *J. Exp. Med.*, 84: 25-36.
- WEILL, P. 1919 Über die leukocyten Elemente der Darmschleimhaut der Säugetiere. *Arch. f. mikr. Anat.*, 93: 1-81.
- WIMSATT, W. A. 1944 An analysis of implantation in the bat, *Myotis lucifugus*. *Am. J. Anat.*, 74: 355-411.
- 1945 The placentation of a vespertilionid bat, *Myotis lucifugus*. *Am. J. Anat.*, 77: 1-51.
- 1948 The nature and distribution of lipoids in the fetal membranes and placenta of the bat, *Myotis lucifugus*, with observations on the mitochondria and Golgi apparatus. *Am. J. Anat.*, 82: 393-468.
- WIMSATT, W. A., AND G. B. WISLOCKI 1947 The placentation of the American shrews, *Blarina brevicauda* and *Sorex fumeus*. *Am. J. Anat.*, 80: 361-436.

- WISLOCKI, G. B., H. BUNTING AND E. W. DEMPSEY 1947 Metachromasia in mammalian tissues and its relationship to mucopolysaccharides. *Am. J. Anat.*, 81: 1-38.
- WISLOCKI, G. B., H. W. DEANE AND E. W. DEMPSEY 1946 The histochemistry of the rodent's placenta. *Am. J. Anat.*, 78: 281-346.
- WISLOCKI, G. B., AND E. W. DEMPSEY 1945 Histochemical reactions of the endometrium in pregnancy. *Am. J. Anat.*, 77: 365-403.
- 1946a Histochemical reactions in the placenta of the cat. *Am. J. Anat.*, 78: 1-45.
- 1946b Histochemical reactions of the placenta of the pig. *Am. J. Anat.*, 78: 181-226.
- 1946c Histochemical age changes in normal and pathological placental villi (Hydatidiform Mole, Eclampsia). *Endocrinol.*, 38: 90-109.
- WISLOCKI, G. B., AND W. A. WIMSATT 1947 Chemical cytology of the placenta of two North American shrews (*Blarina brevicauda* and *Sorex fumeus*). *Am. J. Anat.*, 81: 269-308.
- WOLF, A., E. A. KABAT AND W. NEWMAN 1943 Histochemical studies on tissue enzymes. III. A study of the distribution of acid phosphatases with special references to the nervous system. *Am. J. Path.*, 19: 423-440.
- ZORZOLI, A., AND R. E. STOWELL 1947 Comparison of the distribution of an hexosediphosphatase with glycerophosphatase in different tissues. *Anat. Rec.*, 97: 495-505.

PLATE 1

EXPLANATION OF FIGURES

The preparations of figures 3 to 7 of this plate, and figures 9 and 10 of plate 2 were stained in eosin and methylene blue, but the eosinophilic staining (coinciding with the grey areas) has not been reproduced.

3 Cytoplasmic basophilia in the discoidal placenta of *Pipistrellus subflavus* shortly after implantation. The shell of syncytial trophoblast which caps the embryonic hemisphere of the blastocyst is being invaded by a primary villous sprout ("cytotrophoblastic villus") growing upward from the "basal cytotrophoblast." The endometrium is shown in the upper portion of the figure. See page 70 for full description. $\times 437$.

4 Columnar trophoblastic cells of the subplacental segment of the membranous chorion near mid-gestation (6-mm embryo). Basophilic substance is present everywhere but in the most terminal cytoplasm. Compare with figure 5. The intracellular vacuolar spaces contained lipids. Note that two extravasated maternal erythrocytes are being ingested by the trophoblastic cells near the center of the figure—a phenomenon that is relatively rare in the bat. $\times 437$.

5 Columnar trophoblastic cells of the mesometrial segment of the membranous chorion in late gestation (18-mm fetus). The chorion has shrunk away from its contact with the endometrium. Most of the cytoplasmic basophilic substance has become concentrated in the basal halves of the cells, surrounding the nuclei; the apical halves of most of the cells are intensely acidophilic. The absence of lipid-containing vacuoles is a characteristic of the mesometrial segment of the chorion (Wimsatt, '48). $\times 728$.

6 Basophilia in the discoidal labyrinthine placenta in late gestation (18-mm fetus). The cytoplasmic basophilic substance of the syncytial trophoblastic tubules is concentrated centrally in the syncytium and is separated from the maternal blood space on the one side, and the fetal mesenchyma on the other, by intensely acidophilic zones of the trophoblast. The mesenchymal cells reveal only a very delicate basophilic staining. $\times 728$.

7 Same as figure 6 except that the section was incubated in a solution of ribonuclease before being stained in eosin and methylene blue. The cytoplasmic basophilic substance (ribose nucleic acid) has been completely abolished, but the staining of the nuclei is unaffected. $\times 728$.

8 The distribution of iron in fetal erythroblasts within a vitelline vessel in the yolk sac as revealed by the Turnbull blue reaction (10-mm fetus). $\times 1090$.

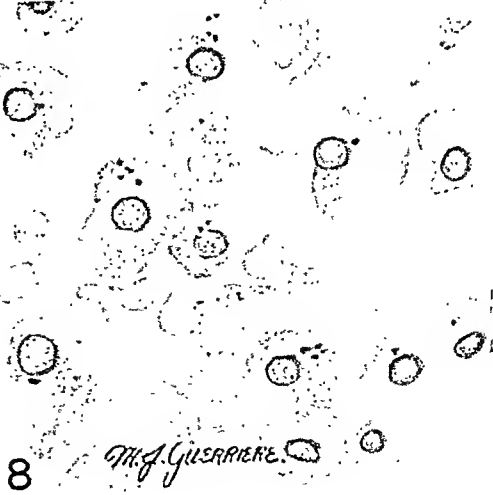
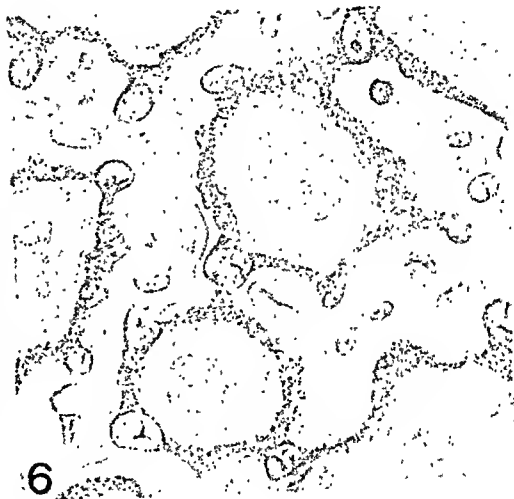


PLATE 2

EXPLANATION OF FIGURES

9 Wall of the vascularized segment of the yolk sac at mid gestation (6-mm embryo). Cytoplasmic basophilia is intense in the entodermal cells, but is very scant in the columnar mesothelial cells of the exocoelomic surface. The basophilic striations in the basal cytoplasm of the entodermal cells are a characteristic of the earlier stages of pregnancy. Compare with figure 10. $\times 728$.

10 Wall of the vascularized segment of the yolk sac near term (18-mm fetus). The entodermal cells are lower, and the cytoplasmic basophilic substance has become concentrated about the nuclei in the apical cytoplasm. It has not appreciably diminished in intensity. This apical concentration of ribose nucleoprotein in the entodermal cells in late pregnancy is unique among the mammals described thus far. In other species it becomes concentrated basally. It is of interest that the polarization of the cells with respect to other inclusions (e.g., the Golgi apparatus) is likewise reversed (Wimsatt, '48). The mesothelial cells show almost no basophilic staining at this late stage. $\times 728$.

11 Section of the placental disc of *Pipistrellus subflavus* at a slightly later stage than that shown in figure 3, and stained by the Turnbull blue reagents. Iron is present as fine granules in the lumen of the maternal blood space, and in the syncytial trophoblast immediately surrounding the space. Traces of iron are also visible in the maternal erythrocytes within the space and in several of the trophoblastic nuclei. The basal cytotrophoblast is shown to the left in the figure and portions of "cytotrophoblastic villi" are shown at the top and at the bottom of the figure. $\times 437$.

12 Turnbull blue reaction in the placental labyrinth of a specimen in late gestation (16-mm fetus). At the top of the figure is shown the uterine wall and the apposed subplacental segment of the membranous chorion. The section was taken laterad to the placental attachment to the basalis. The blue staining iron is principally concentrated in the mesenchymal stroma in the deeper half of the placental disc, being especially conspicuous in the region underlying and surrounding the placental attachment. $\times 36$ (approx.).

13 A portion of the field shown in figure 12 at higher magnification to show the localization of stainable iron in the fetal stroma. Note that it is principally concentrated next to the syncytial trophoblast and shows no preference for the fetal vessels (identified by the larger erythrocytes which they contain). A reaction was not obtained in the syncytium with Turnbull or Prussian blue. $\times 437$.

14 Macallum reaction for "organic" iron in the placental disc during the second half of gestation. A very faint positive reaction characterizes the syncytial trophoblast, but is evident only in the central zone of the syncytium, roughly corresponding to the distribution of ribose nucleic acid in late pregnancy. A red Wratten filter, which blackens and intensifies the light blue reaction, was employed in order to make the drawing, and hence the iron is represented in dark grey shading. All nuclei likewise show a faint positive reaction. $\times 728$.

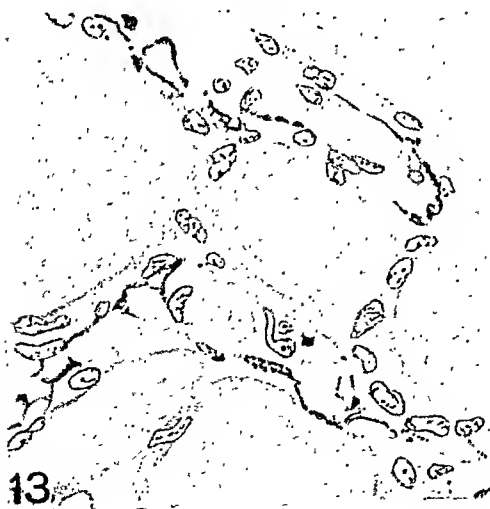
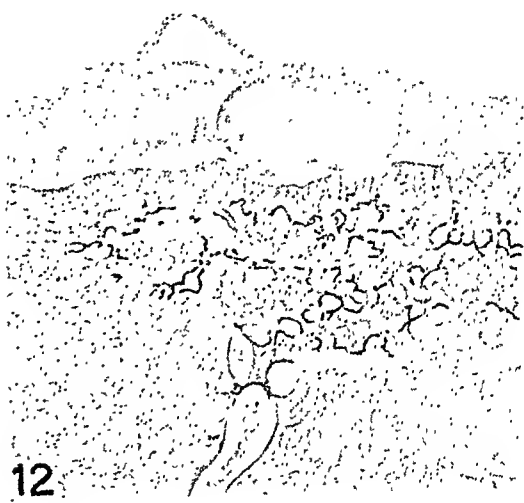


PLATE 3

EXPLANATION OF FIGURES

15 Bauer-Feulgen preparation of the uterus and fetal membranes early in the first half of gestation. An abundance of glycogen is visible in the paraplacental decidual cells above the placental disc, and some in the glands. $\times 24$.

16 Higher magnification of area included in the box in figure 15. The glycogen-rich paraplacental cells are shown at the top of the figure. The irregular spaces below are the maternal blood channels in the syncytial trophoblast. The darkly stained spots between the maternal blood spaces are deposits of glycogen in the cells of the cytotrophoblastic villi, which have not yet been invaded by fetal vascular tissues. $\times 180$.

17 Abembryonic segment of an implanting blastocyst in contact with the decidua parietalis mesometrially. Glycogen is present in both the trophoblastic and endodermal cells of the bilaminar blastocyst (arrow) and in the degenerating glandular cells of the adjoining endometrium. Bauer-Feulgen stain. $\times 437$.

18 Photomicrograph of abembryonic pole of another implanting blastocyst. Glycogen is abundantly present in the columnar trophoblastic cells (which have shrunk away from the endometrium) and in the adjoining connective tissue and epithelial cells of the endometrium. In general glycogen is more abundant in the trophoblast whenever the contiguous endometrium contains it in large amounts. Bauer-Feulgen stain. $\times 180$.

19 Glycogen in the trophoblastic cells of the subplacental membranous chorion at mid-gestation (6-mm embryo). The endometrium lies to the right. Bauer-Feulgen stain. $\times 200$ (approx.).

20 Glycogen in the trophoblastic cells of the subplacental chorion during the second half of gestation (10-mm fetus). The endometrium, containing rich glycogen deposits is at the right. From this period on the amount of glycogen in the chorionic cells declines. During most of gestation glycogen is associated with lipids in the subplacental chorionic cells (Wimsatt, '48). Bauer-Feulgen stain. $\times 325$.

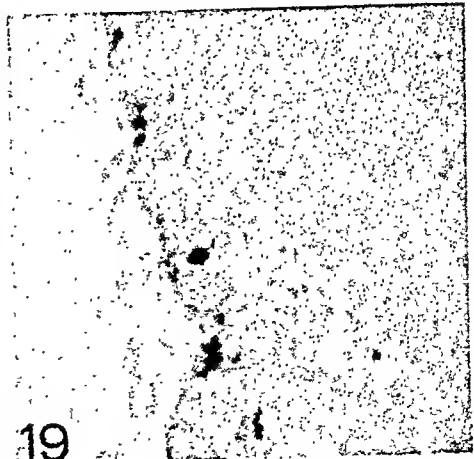
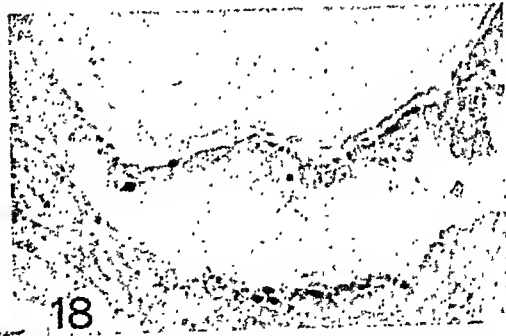
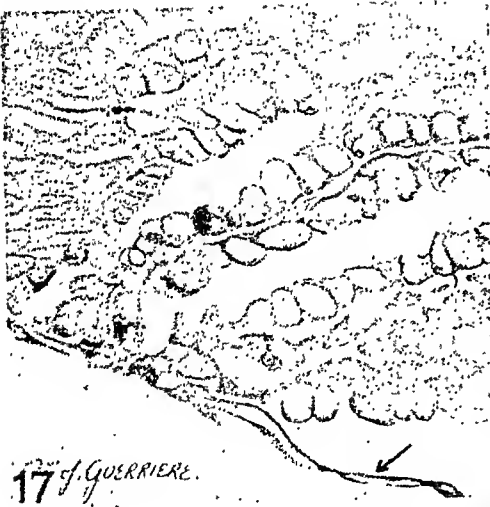
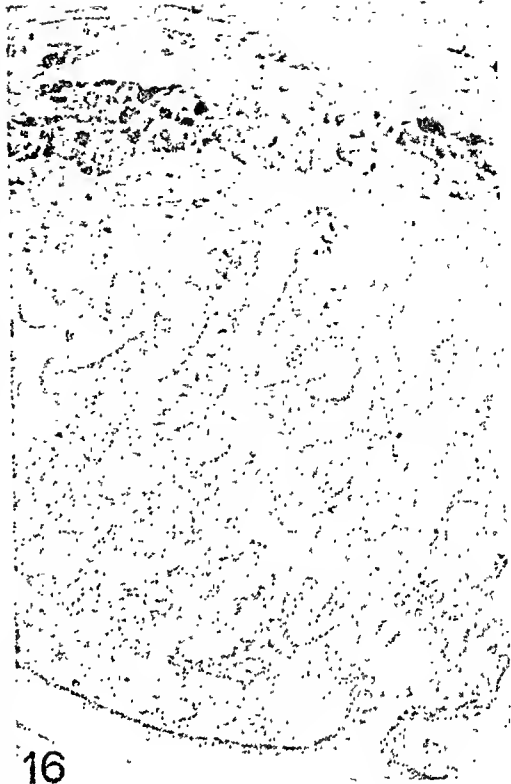
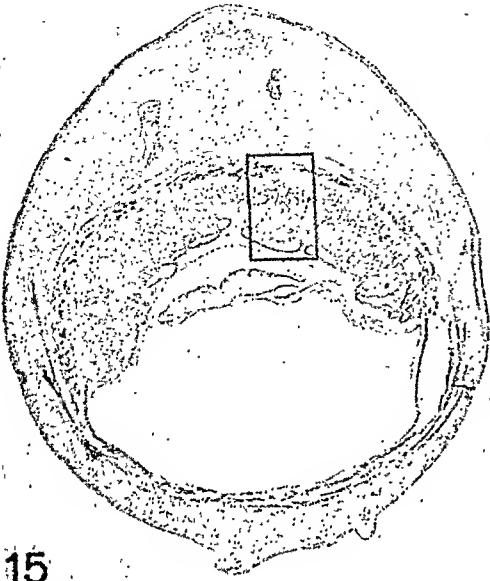


PLATE 4

EXPLANATION OF FIGURES

21 Glycogen in the fetal tissues opposite the decidua parietalis mesometrially at mid-gestation (6-mm embryo). The enlargement of the embryo has invaginated the roof of the yolk sac and reduced its cavity to a crescentic cleft. Glycogen is abundant in the abembryonic trophoblast (adjoining endometrium at top of figure) and in the mesothelium of the yolk sac adjoining the exocoelom. It is present in smaller amounts in the columnar endodermal cells and in the amnion. Bauer-Feulgen stain. am., amnion; end., endometrium; exo., exocoelom; y-s. c., yolk-sac cavity. $\times 325$.

22 Glycogen in the yolk sac late in gestation (16-mm fetus) stained by the silver method of Mitchell and Wislocki ('44). c., yolk-sac cavity. $\times 180$.

23 Glycogen in the yolk sac early in the second half of gestation (10-mm fetus). Note the extensive folding of the exocoelomic surface of the yolk sac. Glycogen is most abundant in the mesothelial cells, but small deposits are also present in most of the endodermal cells. exo., exocoelom; y-s. c., yolk-sac cavity. Silver method of Mitchell and Wislocki ('44). $\times 200$ (approx.).

24 A portion of the placental labyrinth at mid-gestation showing glycogen in the remaining cytotrophoblastic cells. It is absent in the syncytial trophoblast. f. c., fetal capillary; f. s., fetal stroma; M., maternal blood channel. Bauer-Feulgen stain. $\times 325$.

25 Tubo-uterine junction just before the stage of implantation. Observe the extreme vacuolated appearance of the columnar epithelial cells, a condition which presages a breakdown and reorganization of this epithelium. Hematox. and eosin stain. $\times 100$ (approx.).

26 Tubo-uterine junction at a comparable stage to that of figure 25 and showing the massive accumulation of glycogen in the vacuolated epithelial cells. The epithelium is cut tangentially to its surface. Similar, but less extreme changes occur also in the remainder of the oviductal epithelium during the late pregestational period. Bauer-Feulgen stain. $\times 100$ (approx.).

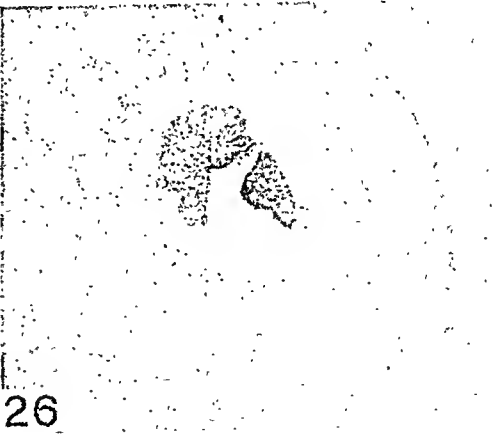
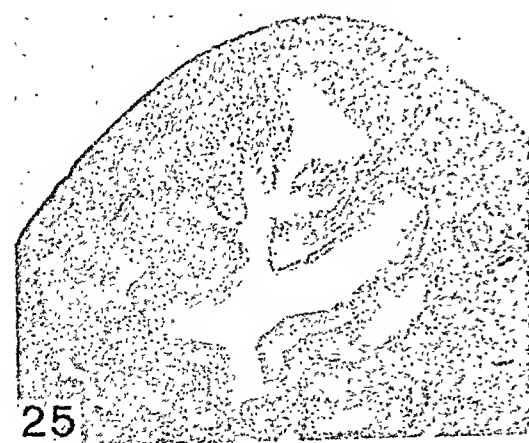
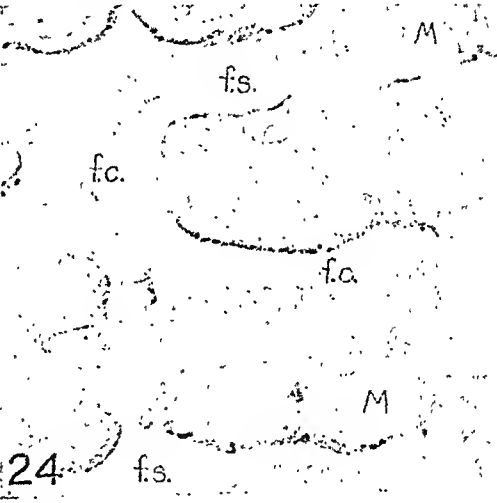
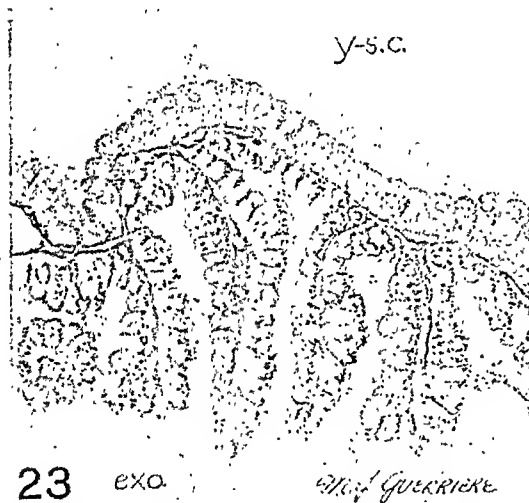
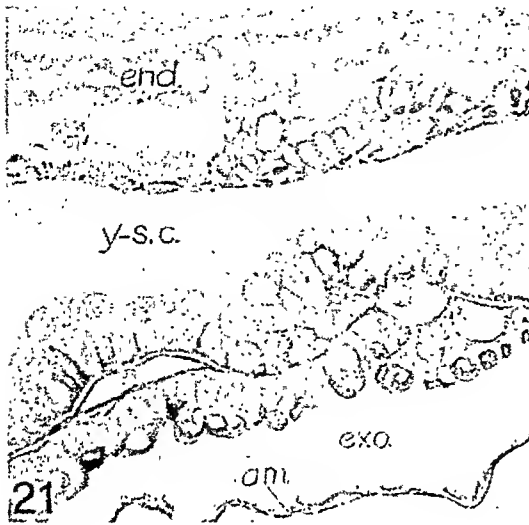


PLATE 5

EXPLANATION OF FIGURES

All of the figures of this plate demonstrate alkaline phosphatase activity (pH 9.4) in the maternal or fetal tissues.

27 Alkaline phosphatase in the uterine and glandular epithelium and in the nuclei of the endometrial connective tissue cells shortly before implantation. Na- β -glycerophosphate as substrate; 4 hrs. incubation. $\times 33$ (approx.).

28 Reconstituted epithelium overlying the parietal decidua mesometrially in late pregnancy (18-mm fetus). Alkaline phosphatase activity is most conspicuous in the apical cytoplasm and in the nuclei. Nucleic acid as substrate; 4 hrs. incubation. $\times 200$ (approx.).

29 Decidua basalis and discoidal placenta near mid-gestation (5-mm embryo). Alkaline phosphatase activity is intense in the myometrium, the decidua basalis, and the amniotic ectoderm (thin line to the far left), but is confined to the nuclei in the paraplacental decidual cells. A moderate reaction characterizes the syncytial tubules of the placental disc. Nucleic acid as substrate; 4 hrs. incubation. d. b., decidua basalis; d. p., discoidal placenta; my., myometrium; ppl. d., paraplacental decidual cells. $\times 32$.

30 Portion of the discoidal placenta (left), subplacental membranous chorion, amnion (lower right corner) and myometrium in late gestation (18-mm fetus). The syncytial tubules show moderate phosphatase activity; activity in the membranous chorion is limited principally to the nuclei of the trophoblastic cells; the amniotic ectoderm and myometrium show intense enzyme activity. Nucleic acid substrate; 4 hrs. incubation. m. ch., membranous chorion; my., myometrium. $\times 32$.

31 Same specimen as in foregoing figure but under higher magnification and with Na- β -glycerophosphate as substrate (4 hrs. incubation). Intense enzyme activity characterizes the periphery of the smooth muscle fibers of the myometrium. The trophoblastic nuclei of the membranous chorion have been stained with paracarmine, but the trophoblastic cells are negative for phosphatase activity. my., myometrium; m. ch., membranous chorion. $\times 250$ (approx.).

32 Yolk sac (left) and amnion (upper right) near term (18-mm fetus). Phosphatase activity in the yolk sac is principally confined to the nuclei. In the amnion an intense reaction is given by the ectodermal layer. Nucleic acid as substrate; 4 hours incubation. $\times 32$.

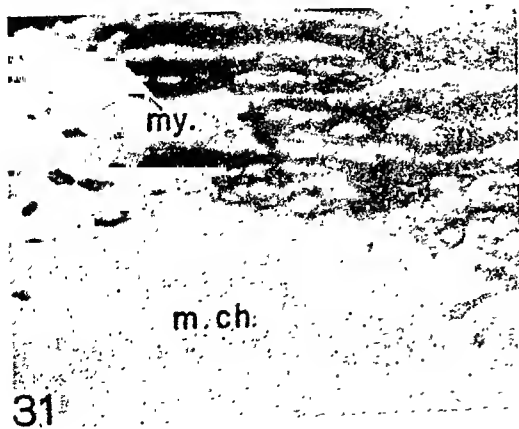
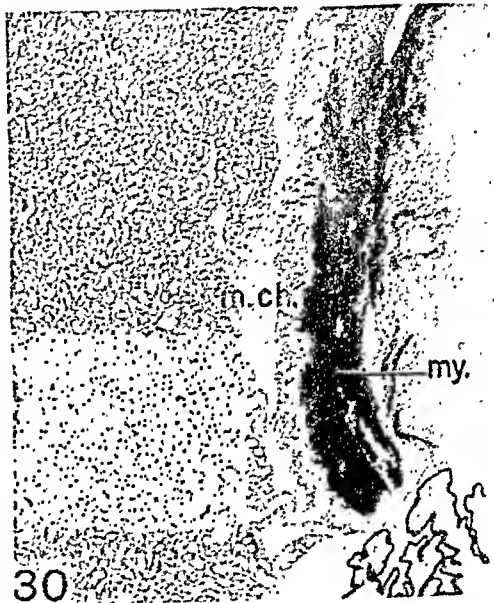
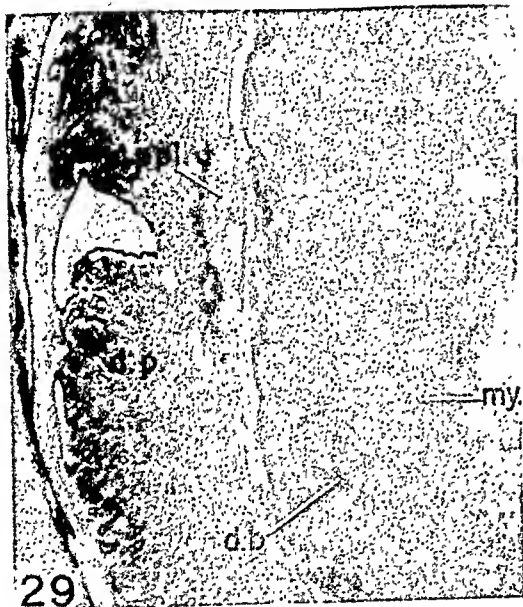


PLATE 6

EXPLANATION OF FIGURES

33 Section through the placental disc shortly after implantation. In the center of the figure a primary cytotrophoblastic villus is pushing into the overlying shell of syncytial trophoblast which already encloses the more superficial maternal capillaries. The maternal endothelium still lines the blood space and shows intense alkaline phosphatase activity. The syncytium also displays intense enzyme activity, but the cytotrophoblastic cells, except those adjoining the syncytium, and the endometrial tissues above are essentially negative. Na- β -glycerophosphate as substrate; 4 hrs. incubation; pH 9.4; paracarmine counterstain; M., maternal blood space; s. e., "swollen endothelium." $\times 437$.

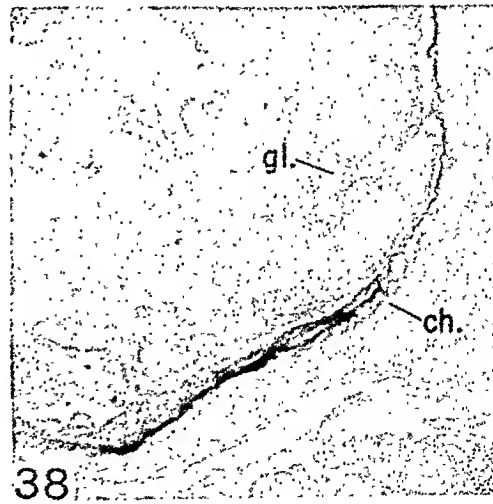
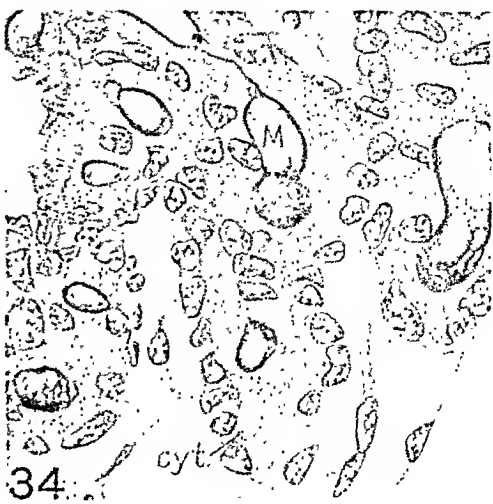
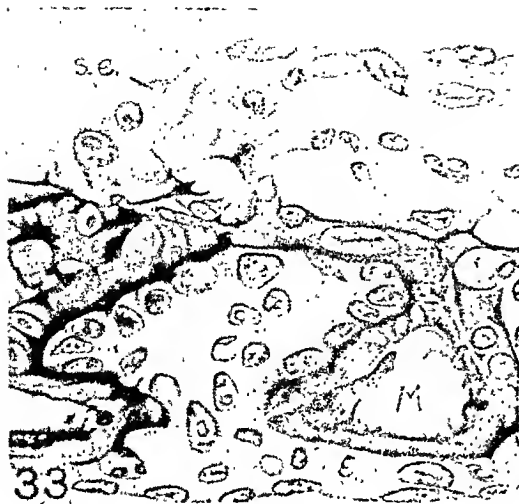
34 Section of placental disc before mid-pregnancy (3-mm embryo) showing alkaline phosphatase activity in the syncytium. The outermost zone of the syncytium adjoining the maternal blood space shows intense enzyme activity, whereas the reaction in the remainder of the syncytium is comparatively weak. The cytotrophoblastic cells adjoining the syncytium are non-reactive. Na- β -glycerophosphate as substrate; 4 hrs. incubation; pH 9.4; paracarmine counterstain. cyt., cytotrophoblast; M., maternal blood space. $\times 437$.

35 Alkaline glycerophosphatase activity in the syncytial trophoblast near mid-gestation (5-mm embryo). Enzyme activity is still intense in the narrow outer zone of the syncytium adjoining the maternal blood space, but seems to have decreased slightly in the remainder of the syncytium. The cytotrophoblast is only irregularly present at this stage. This figure should be compared with figure 36 depicting a specimen in which another substrate was used. Na- β -glycerophosphate as substrate; 4 hrs. incubation; pH 9.4; paracarmine counterstain. M., maternal blood channel. $\times 437$.

36 Alkaline phosphatase activity in the syncytial trophoblast near term (18-mm fetus) with nucleic acid as substrate. Two observations related to enzyme distribution should be noted. First, the intense activity of the outer zone which characterizes the glycerophosphate-treated sections during the first half of gestation is absent following the use of nucleic acid as substrate, both early, and in the late stage here figured. Secondly, the remainder of the syncytium, which shows a relatively low phosphatase activity with glycerophosphate, gives a more intense reaction with nucleic acid, a reaction that is still quite evident at term. Nucleic acid as substrate; 4 hrs. incubation; pH 9.4; paracarmine counterstain. M., maternal blood channel. $\times 437$.

37 Mast cells at the endometrio-myometrial junction (decidua parietalis) near mid-gestation (7-mm embryo). The mast granules are stained metachromatically with toluidine blue. $\times 728$.

38 Glands of the cervical region of the uterus stained by the Bauer-Feulgen method following saliva digestion. The mucoid secretion within the cells, and that which has been discharged into the uterine lumen adjoining the chorion stains intensely reddish. The same material stains metachromatically with toluidine blue. ch., chorion; gl., uterine gland. $\times 67$.



M. J. GUERRIERE

REPRODUCTION OF A SOUTH AMERICAN RODENT, THE MOUNTAIN VISCACHA

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TWELVE FIGURES

This investigation arose from a desire to study in the wild the reproductive habits of some rodent of the superfamily Hystricoidea — an important group in Central and South America but represented in North America only by two species of porcupines. The guinea pig is the only hystricoid in which the reproductive cycle has been investigated in detail, and it has been studied only in captivity. The important fur-bearers, chinchilla and nutria, are hystricoids, as are also capybaras, agoutis, and many other large rodents. For this study a close relative of the chinchilla, the mountain viscacha (*Lagidium peruanum* Meyen), was chosen because large numbers could be obtained easily in a region where I wished to carry on other work.

Viscachas are rabbit-sized, diurnal rodents that live in colonies in the rock slides and cliffs of the Andes. They range between timber line and permanent snow from Peru to southern Chile; in southern Peru, where this study was made, they live from 12,000 to more than 17,000 feet. Most of this work was carried out in a cold, desolate valley at 16,000 feet near the continental divide southeast of the center of Lake Titicaca. A life history of the viscachas in this region will appear in the *Journal of Mammalogy*.

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MATERIAL AND METHODS

Viscachas were collected and observed while I was with the Gardner Peruvian Expedition of the Museum of Comparative Zoology at Harvard University from November 29, 1939, to January 10, 1940, and while with a second expedition from July 14 to December 13, 1946. The fact that this period does not encompass the entire breeding season seriously limits the conclusions that can be drawn regarding some aspects of reproduction, but the studies had to be terminated without settling some matters such as the number of pregnancies experienced yearly by each female, and the exact length of gestation.

Four hundred and ninety-eight viscachas were examined, all but 25 by careful dissection. Three hundred and eighty-nine of the total number were shot, 62 were trapped alive, and 47 live ones were bought from the natives. Almost all of those shot were measured, weighed, and dissected as soon as they were brought into camp. The female reproductive organs were examined in situ and then were preserved in Bonin's fluid. In males the reproductive organs were examined, the testes were measured, and occasionally bits of testis and epididymis were preserved in Bonin's fluid. For use in determining age, we dried the baculum and one humerus from most of the males; one humerus and, in the absence of a baculum, the skull from most of the females. Serial sections of 63 selected pairs of ovaries were cut at 10μ and stained with hematoxylin and eosin. The small pieces of the male reproductive organs were prepared in a similar fashion.

Age was estimated on the basis of body weight in viscachas under two pounds, above this weight by the degree of closure of the epiphysis at the head of the humerus. The skulls were less satisfactory as age indicators.

In addition to spending many days watching wild viscacha colonies, we kept 109 viscachas in captivity from one to 50 days. Most of these did not thrive; some died, some were sacrificed, and 36 were released after they had been weighed

and marked by ear-notching or toe-cutting. Before their release 22 of these had undergone laparotomies so that we could determine the condition of the reproductive organs or remove one ovary. Eleven of these laparotomized, released animals were shot later in the season. Similar operations were also performed on 19 of the animals retained in captivity. The laparotomies were carried out under ether anesthesia and with a "semi-sterile" surgical technique.

Viscachas are excellent surgical patients. They take ether easily; they go under rapidly, once under they require but little additional ether and little attention, and they suffer no after-effects. They bleed so little that hemostats seldom have to be used in laparotomies through the back or belly. Hemorrhage after nicking one ear for identification is frequently greater than that resulting from several laparotomies. No deaths resulted from 41 operations.

I am indebted to George P. Gardner, Jr., David Hertig, Allen Enders, and Anita Pearson for much help in the field, and to Dr. F. L. Hisaw, Dr. A. B. Dawson, and my wife for assistance in planning and completing the work.

OBSERVATIONS

Seasons of breeding

Male viscachas are apparently capable of breeding all year around. The testes of all grown males contained sperm from July through December (fig. 1), and since there were both pregnant females and young viscachas less than three months old present in August, it is probable that the males were capable of breeding from January to June, a period when no collections were made. The fact that all adult male viscachas were in breeding condition (fig. 1) indicates that once a male becomes fertile, he remains so for the rest of his life.

Anatomical evidence and field observations demonstrate a definite peak of breeding activity at the end of October and early in November (fig. 2), resulting in the impregnation of every mature female before mid-December (fig. 2). Some, or

perhaps most, females breed again later in the season, but all are anestrus in September and early October.

Southern Peru is in tropical latitudes and it is not known whether the lengthening of the days in the winter and spring is pronounced enough to serve as a stimulus for breeding of

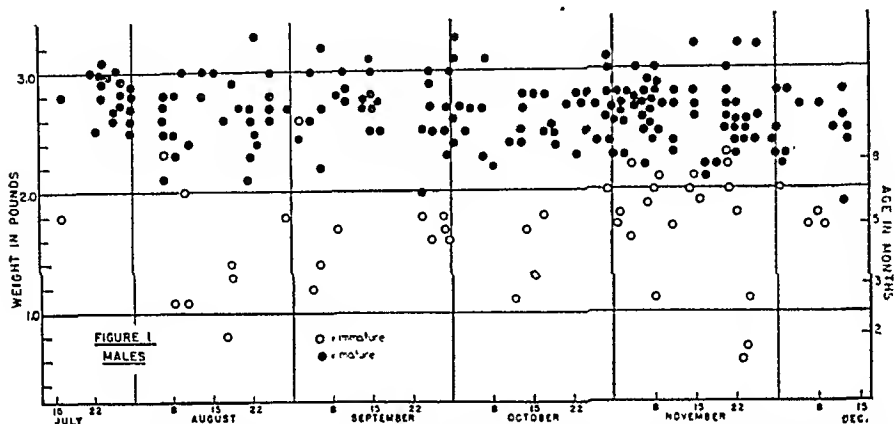


Fig. 1 Weight, age, and sexual condition of wild male viscachas plotted with reference to the date of their capture. Specimens with sperm in the testes were considered mature. A fairly close correlation exists between weight and age up to 8 months (2.2 pounds). The age of viscachas heavier than this cannot be determined from this chart.

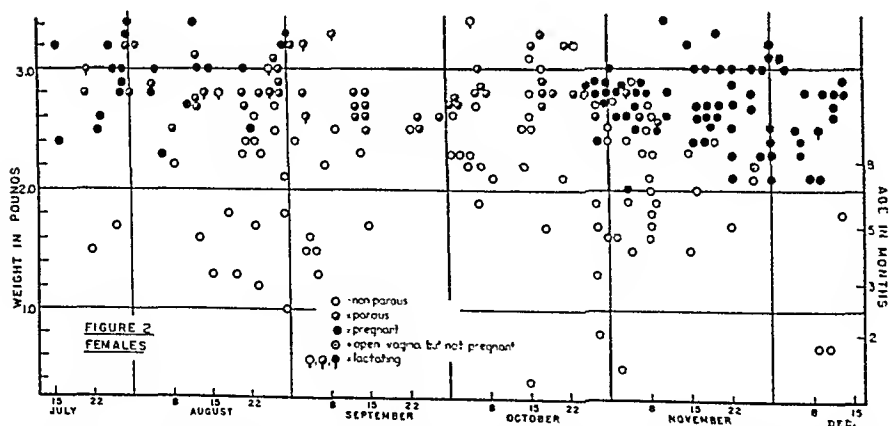


Fig. 2 Weight, age, and sexual condition of wild female viscachas plotted with reference to the date of their capture. Females were judged to be parous if they had large, dark nipples and stout uterine horns. A fairly close correlation exists between weight and age up to 8 months (2.2 pounds). The age of viscachas heavier than this cannot be determined from this chart.

viscachas. Of the dozen or so other kinds of rodents living in the region, all are anestrus in July, August, and early September, and most or all of them breed in October or November. This is true for murid rodents (of Northern Hemisphere descent) as well as for the "native" hystricoids. While living in the region one gets the impression that late September, October, and early November are the spring, despite the imminence of snow and hail from late November to April.

Since the viscacha breeding season begins in mid-October and pregnancy lasts about three months, the principal crop of young is born in the middle of the wet season — presumably a difficult season for an animal whose fur wets easily. Perhaps the greener vegetation at this time compensates for the miserable weather, or perhaps the viscacha has been unable to break away from an inherent predisposition to breed while days are lengthening. The later births seem better timed, for the young emerge into sunny days.

Sexual maturity of males

Sexual maturity usually can be determined from the size of the testes. Microscopic sections were made of small pieces of the testes and epididymides of 16 critical specimens; each testis 18 mm long or larger and the corresponding epididymis contained sperm. Among the 219 males examined, 173 had testes 22 mm and over, 43 had testes 14 mm or smaller, and only three fell between 14 and 22 mm. Consequently, there was seldom difficulty in telling at a glance whether a particular male was sexually mature. Since so few specimens were secured with testes between 14 and 22 mm long, it must be concluded that either such transitional individuals avoid traps and hunters, or that the growth of the testes between these sizes is extremely rapid. The latter explanation is probably the correct one.

Sexual maturity of the viscachas represented in figure 1 was judged in most cases by the size of the testes. All cases

with borderline measurements were determined by examining sections of the testes. It may be seen that body-weight is a fairly reliable index of sexual maturity. Only one individual weighing less than two pounds was mature, and only one weighing more than 2.3 pounds was immature. Using weight and epiphyseal closure to estimate age, it appears that males reach sexual maturity at about 7 months of age regardless of the season, and remain fertile for the rest of their lives (fig. 1 and Pearson, in press).

Sexual maturity of females

It may be seen in figure 2 that some females as small as two pounds become pregnant, but only during the more active part of the breeding season. Young females that reach two pounds during the non-breeding season (August and September) differ from males of similar age and weight in that they do not come into breeding condition immediately but mature sexually during the breeding season in late October or November, at which time they may weigh 2.5 pounds and occasionally more.

The youngest breeding females were a little more than 6 months old (fig. 2). These were probably young individuals that happened to be approaching the minimum age for reproduction at the peak of the breeding season. There is no evidence of reproductive failure in old individuals.

Male anatomy

The testes are ellipsoidal, as much as 37 mm long, and are usually found in the abdominal cavity. A thin-walled scrotal pouch is visible on each side of the pubic symphysis and usually contains the convoluted tail of the epididymis. The testes can be pushed caudad part way into these pouches, but the latter are not large enough to contain an entire testis.

The most conspicuous of the accessory glands are the two seminal vesicles. In adult males each vesicle is an irregularly

curved tube about 100 mm long, from one side of which project finger-like sacculations. In some individuals the vesicles reach from the base of the bladder almost to the kidneys, and each contains more than 5 cm³ of milky fluid. The prostate glands are paired structures lying dorsal to the seminal vesicles. The prostatic ducts enter the urethra distal to those of the seminal vesicles and proximal to the ducts of the bulbo-urethral (Cowper's) glands. Each bulbo-urethral gland is oval, about 14 mm long, and lies subcutaneously at the base of the tail. The ducts run anteriorly and ventrally to enter the urethra at the caudal end of the pubic symphysis.

The penis extends craniad for about 40 mm from the crura, then bends sharply caudad for 30 mm. When erect it is bright pink and projects anteriorly for about 65 mm. The glans is covered with small spines and is supported by a club-shaped baculum as much as 25 mm long in old individuals.

Vaginal plugs

As in some opossums, bats, insectivores, and in many rodents, a hard waxy plug forms in the vagina of the viscacha after copulation. Among the close relatives of the viscacha, such a plug has been described by numerous authors for the guinea pig, by Camus and Gley ('22) for the plains viscacha (they called their animal *Viscacia viscacia*, but from the weights and locality they must have had *Lagostomus*), and a photograph of a microscopic section of a plug from *Chinchilla* has been published by Asdell ('46). Engle ('26) showed that the guinea-pig plug was formed by the action of a secretion of the proximal lobe of the prostate on the fluid from the seminal vesicles. Camus and Gley demonstrated likewise that prostatic fluid added to the vesicular fluid of the plains viscacha formed a waxy solid, and that fluid from Cowper's gland failed to do this.

In one experiment with *Lagidium* I obtained wax formation by mixing fluid from the seminal vesicles, prostate, and bulbo-urethral glands. I obtained no reaction from mixing only prostatic and vesicular fluid, but it is possible that I did not

use the proximal lobe of the prostate, to which the necessary enzyme may be restricted, as in the guinea pig. It is certain that secretions from the female are not necessary to form a plug because I frequently found waxy casts of the urethra and preputial region protruding from the penises of males that had been shot.

Vaginal plugs of the mountain viscacha are white, finger-like casts about 17 mm in diameter and 40 mm long, moist and firm while still in the vagina, but tough, dry, and shrivelled after they have been expelled. They expand the vagina considerably while they are in situ. Impressions of sizeable rugae are visible on the plugs when they are examined closely, and a small tab near the center of the anterior end marks the opening in the cervix. As in the chinchilla a rather tough film of cornified vaginal epithelium envelops the plug when it is expelled. Of the 16 females collected that had open vaginas but no implantation swellings in the uterus, only two contained complete vaginal plugs, and a third had traces of a plug stuck in the fornices and cervix. Because so few of these females contained plugs, it must be concluded that the plugs remain in the vagina for only a short time after copulation. Two of these females containing vaginal plugs had ovulated recently and the third was approaching ovulation.

The reproductive cycle of the female

The uterus of the viscacha is Y-shaped, composed essentially of two long horns which merge posteriorly into a common body in which the lumina remain distinct for a considerable distance. In an early-pregnant specimen the lumina remained separate for 15 mm posterior to the point where the two horns joined, but a common lumen extended for 5 mm from the posterior tip of the partition separating the two lumina to the os cervix.

The horns of the uterus are very slender in immature females, but become stouter at the approach of estrus. Despite the fact that implantation occurs almost always in the right

horn, both horns are the same size in nulliparous individuals, and both become thin-walled and enormously swollen with fluid at estrus. Only three specimens were obtained with the uterus in this advanced stage of distention, so the condition probably lasts no more than a day. Two of these females contained vaginal plugs, and the vagina had not quite opened to the outside in the third. Only one of the three had ovulated (one of those with a vaginal plug); an ovum was found upon sectioning the middle third of the oviduct. The fluid in the distended uterus of the unmated specimen at estrus was recorded in my field notes as clear and mucilaginous. In the female that had ovulated the fluid was described as having the appearance of milk of magnesia, but somewhat slimy; it could be drawn out into a thread 15 mm long. In this specimen it was abounding with sperm. It is interesting to note that the fluid can be sloshed about in the uterine horns without escaping either through the cervix or the oviducts, and it passes readily through the corpus uteri from one horn to the other.

The vagina is very slender in immature females, but begins to broaden a few weeks before the first estrus. A few days before breeding takes place, the vaginal walls become quite thick and tough; considerable regression occurs in early pregnancy. The vagina opens through a transverse slit between the urinary papilla and the anus, but this slit is open only for a few days at estrus and for a few days at parturition. It may be smoothly closed or scabby during early pregnancy, but in most females containing embryos smaller than 25 mm it can be reopened by gentle traction of the skin in front of and behind the slit.

Each ovary is only partially enclosed in a bursa, the opening of which is so large that the entire ovary can be pushed through it. Both ovaries are elongate in an antero-posterior direction and are closely attached not by an ordinary stem and hilus but by a mesentery stretching the entire length of the ovary. This mesentery usually extends from one edge of the flat or prismatic ovaries. Even large follicles and corpora

lutea scarcely protrude from the surface of the ovary. The left ovary is frequently very thin or flat, and sometimes appears to be only a slight thickening of the long attaching mesentery. Long, deep longitudinal fissures are common, especially in the left ovary.

The ovaries are rather small for an animal of this size. The average weight of an ovary, determined from a series of 45 parous, pregnant, estrous, and proestrous viscachas, was 22.9 mg. The right ovary was almost always heavier than the left in adults, a fact of some importance in relation to the problem of unilateral gestation. The right ovary averaged 32.7 mg (7.5-134.6), and the left 13.1 mg (0-44.6). In one case the left ovary was so small it could not be located, and in several others it weighed less than 4 mg. In only one case did the left ovary weigh more than the right. In this individual both the corpus luteum and the fetus were on the left side (left ovary, 32.6 mg; right ovary, 25.4 mg). In young viscachas both ovaries are about the same size, and it is not until about two weeks before the first breeding that the right ovary becomes larger. As a rule it remains larger than the left for the rest of the life of the animal. The right ovary reaches its greatest weight during the second half of pregnancy when it contains more corpora lutea and large follicles than at other times. The left ovary, despite its comparatively functionless condition, is heaviest at this time also.

Ovulation is almost always from the right ovary. That it occurs near the time of copulation is demonstrated by three specimens that contained vaginal plugs: two of these females had ovulated and one had not. Only one follicle ruptures at each heat period and most, if not all, females become pregnant at the first ovulation, for no early-pregnant female had more than one corpus luteum and no female was found with a corpus luteum but without an embryo. Some follicles become as large as 1.4 mm before rupturing. I have been unable to determine whether ovulation is spontaneous or is induced by copulation.

The medium-sized follicles that fail to rupture tend to disappear within a few days after copulation, leaving the

ovary with a new corpus luteum and only small follicles. However, by the time a swelling is visible in the uterus (about 11 days after ovulation), medium-sized follicles have reappeared and these continue to grow to full pre-ovulatory size and become luteinized when the embryo is as small as 19 mm C-R. Despite the presence of a large, apparently functional, primary corpus luteum, this process of development and luteinization of large follicles continues until there may be a dozen secondary corpora lutea in the right ovary. In general it may be said that the more advanced the embryo, the more corpora lutea will be found in the right ovary. I have searched serial sections of many of these secondary corpora lutea for a contained ovum. Sometimes I have found one, sometimes not; never have I found a ruptured large "secondary" follicle, although many have appeared to be about to rupture. I can only conclude that sometimes, perhaps always, these follicles become luteinized without rupturing. In either event they form apparently functional corpora lutea which are sometimes almost as large (but usually only half as big) as the primary corpus. The luteal cells of the secondary corpora, after these organs have had time to develop fully, are indistinguishable from those of the primary corpus.

Cole et al. ('31) have reported that several accessory corpora lutea are formed in the mare between the 40th and 150th day of pregnancy, and that ovulation apparently occurs in some of these cases. In the rat small corpora lutea form at 4- or 5-day intervals during pregnancy (Swezy and Evans, '30; Evans and Swezy, '31). No eggs were found in the oviducts of these rats, although comparatively few of the corpora showed a retained ovum.

The corpora lutea are never a very conspicuous feature of the viscacha ovary when it is viewed grossly. Even the primary corpus luteum at its greatest development scarcely projects from the surface of the ovary and is usually visible only as a faint peach-colored discoloration of the surface. In the mare the primary and secondary corpora disappear before parturition, but in viscachas they remain as small

degenerate structures when the young are born. Judging from their reduced diameters and shrunken cells it seems unlikely that any of the corpora are functional near the end of pregnancy, and they disappear completely before lactation ceases. When the corpora cease to function is not known, although we have the following evidence that they may not be necessary to maintain pregnancy.

On December 7 the right ovary containing the corpus luteum was removed from a female with a 17-mm swelling (indicating a pregnancy of about 40 days) in the right cornu. Then she was released. The swelling had grown to 19 mm and the 15-mm embryo appeared to be healthy when the mother was shot on December 12. Although she was shot at the latest possible date, the evening before our departure, only 5 days had elapsed since the right ovary had been removed — too short a time on which to base definite conclusions.

At the end of pregnancy the ovaries may contain large follicles, up to 1.4 mm, but from July to December post-parturitional breeding is not the rule. Only one definite case was encountered: a female containing a 20-mm embryo was shot on December 11 while nursing a 0.7-pound baby. It is not unlikely, however, that early parturition is normally followed immediately by heat, but that no heat follows parturition later in the breeding season: in June or July, for example.

I have no specimens from January to July, so cannot state definitely how many young are produced each year by each female. It may be seen from inspection of figure 2 that a very definite breeding season occurs in October and November resulting in the impregnation of every adult female before the first of December. The young of this pregnancy would probably be born toward the end of January or in February. The problem then presents itself: how many young are produced before the non-reproductive period extending from late August to early October? Assuming that there is post-parturitional heat and a gestation of three months, two more young could be born before August; or assuming a three-month anestrus between pregnancies, only one more could

be born. That most females bear more than one young each year is suggested by the fact that the average viscacha is short-lived. Half the population is less than one year old (Pearson, in press). The production of only one young annually by each adult female would hardly permit such a rapid turnover.

Unilateral pregnancy

Serial sections were made of 32 pairs of ovaries from which an ovum had been released, and in 31 of these cases the single ovulation was from the right ovary. Judging from gross inspection of many other ovaries and from ovarian weights it seems doubtful that the left ovary had experienced ovulation in any of an additional 47 pregnant females whose ovaries were not sectioned.

Every pregnant viscacha had only one embryo, and exclusive of those from which the right ovary had been surgically removed, only two of 72 viscachas with macroscopic implantation sites carried embryos in the left horn of the uterus. In one of these cases the left ovary was larger than the right and contained a single large corpus luteum; there was no corpus luteum in the right ovary. In the other specimen with an embryo in the left horn, the right ovary was larger and contained the corpus luteum, so the ovum must have migrated into the left horn from the right ovary. This transfer may have been across the abdominal cavity, for the ovarian capsules are widely open, or through the corpus uteri. The structure of the corpus uteri is such that it would obviously discourage transmigration, but since uterine fluid can pass readily from one horn to the other at estrus, it is not unlikely that an occasional blastocyst could also pass. Transmigration does not occur in the fur seal (Enders et al., '46), which has almost exactly the same anatomical relationships as the viscacha in the region of the corpus uteri, nor did transmigration of ova occur in 25 guinea pigs from which one ovary had been removed (Kinney, '23).

TABLE I
*Non-pregnant vireochas from which the right ovary was removed. All were released after the operation and were collected
 17 to 55 days later*

| No. | RIGHT OVARY | | | LEFT OVARY | | | CONDITION OF FEMALE |
|------------------|--------------|---------|----------------------------|------------|---------|--|---|
| | Date removed | Weight | Condition | Date | Weight | Condition | |
| 622 | Oct. 23 | 25.1 mg | Follicles up to 0.7 mm | Dec. 9 | 18.5 mg | One large corpus and two large follicles | Pregnant in left horn, embryo 8.5 mm |
| 591 | Oct. 16 | 20.3 mg | Small and medium follicles | Dec. 8 | 20.9 mg | One large, young corpus luteum | Pregnant? Oviduct and uterus not sectioned |
| 588 ¹ | Oct. 15 | 5.5 mg | No large follicles | Dec. 7 | 4.3 mg | Small follicles | Uteri slender. No compensatory growth of left ovary |

¹ 2.2-pound immature female when first caught on October 15. Still had traces of juvenile pelage on December 7.

Effect of removing right ovary. The right ovary was removed from 6 non-pregnant, captive viscachas before they were released. Three of these were recovered about 7 weeks later, and it may be seen in table 1 that the left ovary had undergone ovulation in two of them. Indeed, one of them had a healthy, 8.5-mm embryo in the left horn. The other had ovulated only recently and probably had an ovum or very young embryo in the oviduct or uterus. The third viscacha was immature when ovariectomized and was still immature when recovered: there was no compensatory hypertrophy of the left ovary in this case. The right ovary was also removed from a 7th non-pregnant viscacha which was then kept captive for three weeks and killed on September 16 — about a month before the beginning of the breeding season. The left ovary was small, with no large follicles, although primordial follicles were abundant.

A survey of unilateral pregnancy. In view of the occasional occurrence of natural left-side ovulations and implantations, and in view of the induced left-horn pregnancies described above, it appears that the organs on the left side are entirely capable of functioning; the problem is, "why do they fail to?"

As an approach to this problem I have attempted to assemble in table 2 comparative data bearing on unilateral ovulation and implantation. Four kinds of ovarian activity are found among the animals listed: ovaries equal, equal but alternate, right ovary dominant, and left ovary dominant. Several combinations of uterine function are associated with these ovarian conditions. In animals like the viscacha one uterine horn *appears* to be dominant (i.e., more frequently used), but this probably results from the fact that implantation tends to occur on the same side as ovulation. It is difficult, therefore, to dissociate uterine dominance from ovarian dominance. In some cases this is possible, however: in *Vespertilio murina* only 56% of the ovulations are on the right side, yet implantation is always in the right horn. The right uterus is clearly dominant despite bilateral ovulation. In this respect the most curious animal of all is the bat *Miniopterus natalensis*, for

TABLE 2
A survey of unilateral pregnancy

| CONDITION | AUTHORITY | REMARKS |
|--|---------------------------|--|
| <i>I Ovaries equal:</i> | | |
| <i>a. Uteri equal</i> | | |
| Echidna | Flynn and Hill, '39 | |
| Common shrew (<i>Sorex araneus</i>) | Brumbell, '35 | |
| Shrew (<i>Sorex brevicauda</i>) | Pearson (unpub.) | Right ovary 47% |
| Brown bat (<i>Eptesicus fuscus</i>) | Wimsatt, '41 | Right ovary 51% |
| Rhesus monkey | Hartman, '32 | |
| White rat | Ardi, '20 | |
| Guinea pig | Ibsen, '28 | Right uterus 49% |
| Rabbit (<i>Oryctolagus</i>) | Hammond and Marshall, '25 | |
| <i>b. Right horn dominant</i> | | |
| Little brown bat (<i>Myotis lucifugus</i>) | Wimsatt, '44 | |
| Bat (<i>Myotis myotis</i>) | Duval, 1895 | Right ovary 48% |
| Bat (<i>Pipistrellus pipistrellus</i>) | Deaneasy and Warwick, '39 | |
| <i>c. Left horn dominant</i> | | |
| None known | | |
| <i>II Ovaries equal but alternate:</i> | | |
| Rat kangaroo (<i>Deutongia canaliculus</i>) | Flynn, '30 | |
| Wur seal (<i>Callorhinus ursinus</i>) | Emlers et al., '46 | Uteri probably have equal potentialities |
| Hyena (<i>Crocuta crocuta</i>) | Mathews, '39 | |
| <i>III Right ovary dominant:</i> | | |
| Garter snake (<i>Thamnophis</i>) | Giesdak, '45 | Right ovary 58%. (Right testis larger also) |
| Horse-shoe bat (<i>Rhinolophus hipposideros</i>) | Mathews, '37 | Right ovary 100% |
| Horse-shoe bat (<i>Rhinolophus ferrum-equinum</i>) | Mathews, '37 | Right ovary 100% |
| Bat (<i>Miniopterus australis</i>) | Baker and Bird, '36 | |
| Bat (<i>Miniopterus schreibersii</i>) | Courrier, '27 | |
| Bat (<i>Tadarida cynocephala</i>) | Sherman, '37 | Right ovary 100% |
| Visaccha (<i>Lagidium</i>) | Pearson, n. 13 | Right ovary 98% |
| Cow | Reece and Turner, '38 | Right ovary 60% |
| Ewe | Henning, '39 | Right ovary 59% |
| <i>IV Left ovary dominant:</i> | | |
| Platypus | Flynn and Hill, '39 | Left ovary 100% |
| Birds | | Only left ovary present in most birds, both in some hawks |
| Bat (<i>Miniopterus natalensis</i>) | Mathews, '41 | Right cases, all implanted in right horn, all ovulated from left ovary |
| Mano | Andrews and McKenzie, '41 | Left ovary 60% |
| Sow | Warwick, '26 | Left ovary 55%, fetuses equally distributed |

in the 8 known cases ovulation was from the left ovary and implantation in the right horn. In no mammalian species is the left uterine horn known to be dominant.

It appears from the table that ovarian and uterine function are remarkably susceptible to genetic modification. Right-side, left-side, bilateral, and alternate-side reproduction all appear to be satisfactory in maintaining one species or another, but it will be noted that among mammals the species that have tended towards unilateral or alternate reproduction are those that can survive with a low birth rate; for example, bats, fur seals, and viscachas. These also happen to be species that bear relatively large young.

I have found no satisfactory explanation of why the left ovary in viscachas does not function, nor of why the right ovary does. Until a few weeks before the first estrus the left ovary is as large as the right. It abounds with primordial and small follicles, appears to be as vascular as the right, and is entirely capable of ripening and liberating an ovum, developing a corpus luteum, and maintaining pregnancy—if the right ovary is removed. I have found no important histological difference between the right and left ovaries when their destinies diverge a few weeks before estrus. The follicles in the right ovary grow to ovulating size while those in the left remain small. The left ovary, like the right, increases in size during pregnancy, but only rarely are secondary corpora formed in the left (figs. 7–12). If it is an embryonic preordination that endows the tissue of the right ovary with this special property, it is of interest that both testes of the male viscacha seem to be endowed equally. Perhaps the explanation lies buried with the secret of how the fur seal can ovulate from alternate ovaries each year.

Growth of embryos

Direct measurements were made of the growth of the embryos in 4 viscachas that were examined by laparotomy, released, and subsequently recaptured (table 3). It may be

seen that in the case of no. 622, an embryo 8.5 mm long (C-R) developed in less than 47 days. Since a week or more was probably required for the left ovary to ripen an ovum after the right ovary had been removed, it is probably safe to say that this embryo developed in less than 40 days. Less than 23 days were required for a 3-mm embryo to develop from the small uterine-swelling stage, and exactly 23 days for a 13-mm embryo to develop from the microscopic cluster of embryonic cells found in a uterine swelling 10×12 mm.

TABLE 3
Growth of embryos, calculated from released viscachas

| NO. | EXAMINED | CONDITION | RELEASED | LAPAR | CONDITION |
|-----|----------|---|----------|---------|--|
| 622 | Oct. 23 | Not preg. Rt. ovary removed. One med. follicle (0.7 mm) | Dec. 9 | 47 days | 8.5 mm embryo (uterine swelling 15×18) |
| 780 | Nov. 19 | Possibly a just-visible swelling | Dec. 12 | 23 days | 3-mm embryo (swelling 16×14) |
| 779 | Nov. 19 | Swelling 10×12 mm | Dec. 12 | 23 days | 13-mm embryo (swelling 20×20) |
| 854 | Dec. 7 | Swelling 17 mm | Dec. 12 | 5 days | Swelling 19 mm (embryo 15 mm) |

A less direct method of measuring embryonic growth rates is that of dating specimens from an estimated breeding date. This method can be used only with species in which the beginning of the breeding season is rather abrupt, as among viscachas. A surge of breeding activity about October 26 makes a fairly satisfactory reference base, and I have used this date when making the estimates in table 4. Many individuals of each size group were used for each of the estimates.

Upon combining the data in tables 3 and 4 it appears that the ovum reaches the uterus, becomes attached, and begins formation of the extra-embryonic structures at a rate compa-

table with that found in many other mammals (11 days), but growth of the embryo itself is so slow at these early stages that it is still a 1-mm filmy oval at 23 days (when a rat would already have produced an entire litter), and it has scarcely passed the primitive streak stage (fig. 5) at 32 days (when a rabbit would have finished gestation). Growth beyond the 3-mm stage seems to be at a more usual rate, but at least a month, more likely two months, would be necessary to complete the development of the fetus, which weighs nearly a half pound at birth. The longer of these intervals would result in a gestation of about 100 days. Pregnancy in the closely related chinchilla lasts about 108 days (Dennler, '40).

TABLE 4

Growth of embryos, timed from an estimated breeding date

| GROWTH | ESTIMATED TIME |
|---|-------------------|
| Ovulation to a small uterine swelling, 8-13 mm (embryo still invisible under a hand lens) | 11 days |
| Ovulation to a 1-mm C-R embryo | 23 days |
| Ovulation to a 3-mm C-R embryo | 38 days |
| Ovulation to a 15-mm C-R embryo | 46 days |

In the guinea pig, which is the only other hystricoid for which detailed growth data are available, embryonic growth is similarly slow in the early stages. Ibsen ('28) showed that guinea pig embryos weigh less than half a gram on the 25th day of gestation and that rapid growth toward the 100-gm birth weight at the 68th day does not begin until after the 30th day, when the embryos are about 20 mm C-R.

It was shown above that embryonic growth of the viscacha is extremely slow until the embryo has reached a length of about 3 mm. In the early stages most of the growing potential seems to be directed toward the development of extra-embryonic tissues. The amnion is completed, a chorionic sac about 10 mm in diameter and filled with crystal-clear fluid develops, and a thick placenta has been established before the

embryo itself has reached 5 mm in length. The embryo does not seem to exert itself while all these preparations for its own development are being made, but when the extra-embryonic structures have expanded the uterine swelling to a width of about 15 mm the embryo begins to grow considerably faster than the surrounding structures. This differential growth rate is clearly shown in figure 3 where the crown-rump length of the embryo is plotted against the size of the uterine swelling. It

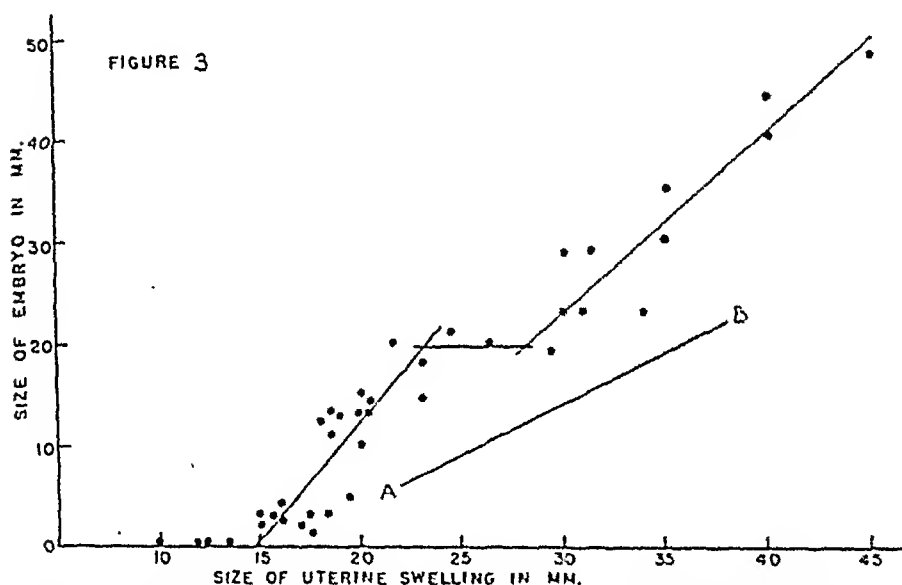


Fig. 3 Relation between growth of the embryo and growth of the extra-embryonic structures. The slope of the line AB represents equal rate of linear growth of the embryo and the extra-embryonic structures.

should be kept in mind that growth is not being plotted against time. An average of the length and width of the uterine swelling was used for the measurement plotted along the horizontal axis of the graph. It may be seen that the embryo does not grow appreciably until the swelling has reached nearly 15 mm, after which the embryo grows more rapidly than the swelling. This is indicated by the slope of the line extending from 15 to 23 mm uterine size. If the embryo and the uterine swelling grew at the same rate, the slope would

be the same as that of the reference line AB. Curiously enough, when the embryo has become almost as large as the swelling, the embryo appears to slow down or even stop growing (relatively) for a time, represented by the horizontal line from 23 to 28 mm, and then resumes growth at a slightly faster rate than the uterine swelling. Perhaps it is closer to the truth to think of the embryo as growing at a constant rate while the uterine swelling changes its growth rate. For example, a sudden increase in the amount of amniotic fluid when the embryo is about 23 mm long would cause an apparent flattening of the growth curve as in figure 3. Making use of the data given by Ibsen ('28), I can find no similar discontinuity in the growth curve of guinea pig embryos. Indeed, he showed that there is a *resorption* of amniotic fluid on about the 55th day, which causes a discontinuity in the *opposite* direction.

Development of the fetus from ovum to birth may be traced in table 5. The full-term fetus with a body half as long as its mother's and weighing nearly half a pound is in accord with the hystricoid tradition of large, well-developed offspring. The ilio-sacral union of the mother remains unossified, which allows some rotation of the pelvis, as in the rabbit, and facilitates the birth of such a large fetus. The pubic symphysis also is unossified but even in late-pregnant specimens it does not become as "relaxed" as that of the guinea pig. Both the ilio-sacral and pubic symphyses appeared to be unossified also in two adult male viscachas.

Lactation

The single pair of elongate nipples is situated on the sides of the thorax (fig. 4), and at the height of lactation the mammary tissue underlying each of these is an oval about 10 cm long. The young viscachas nurse for more than a month after birth, but it is probable that they do not require milk. No viscacha collected, no matter how young, had more than a trace of milk in its stomach, and all had eaten vegetable matter.

TABLE 5
An outline of embryonic development

| SIZE OF EMBRYO (C-H) | SIZE OF UTERINE SWELLING | DEGREE OF DEVELOPMENT | SIZE OF CHORIONIC SAC | SIZE OF PLACENTA | REMARKS |
|----------------------|--------------------------|--|-----------------------|------------------|--|
| mm | mm | | mm | mm | |
| | | Fertilized ovum in oviduct | | | 55 × 81 μ including zona. Boutin's fixative |
| | | Developing ovum in oviduct 50-100 cells | | | 37 × 52 μ including zona Serosa clear |
| 2.5 | 12 × 15 | Embryo not visible with a hand lens | 6 | | Serosa clear. See figure 5 |
| | 14 × 18 | Embryonic disc 2.5 mm, with primitive streak. Allantois 2.5 mm | 9 | | Serosa clear |
| 5.2 | 16 × 18 | Limb buds | 10 × 12 | 7 × 9 | Serosa becoming vascular |
| 11 | 17 × 20 | Eyes, limb buds, no digits | 18 | 16 | Serosa rather vascular |
| 20 | 24 × 29 | Digits and vibrissal papillae | 22 | 21 | Serosa rather vascular |
| 29 | 26 × 34 | Claws, ear pinnae | 20 × 31 | 22 | Serosa rather vascular |
| 48 | 55 × 35 | Vibrissae emerging, no tail hairs | | | |
| 70 | | Vibrissae. No hair except on tail. Total length, 152; tail, 68; hind foot, 27; ear from notch, 14 mm | | | |
| 80 | | Long vibrissae and tail hair; body faintly haired, sharply bicolored, gray on back, pink-white on belly. Eyes not open. 20.5 × 9.7 × 3.9 × 2.3. | | | |
| | | Well-haired, eyes open, testes 8 mm. 32.7 × 15.0 × 6.0 × 4.6. Placenta, 50 × 40 × 16, separates easily from uterus. | | | |
| | | Well-haired, eyes open. 33.0 × 17.0 × 6.0 × 4.9. These last two fetuses must have been full term because a young trapped viscacha had the following measurements: 31.0 × 15.3 × 6.3 × 4.9. | | | |

Lactation has been discussed more completely in another article that also correlates the behavior of wild viscachas with the condition of their reproductive organs (Pearson, in press).

SUMMARY

This study was based on observations of living viscachas both wild and captive, and on dissections of 473 viscachas collected from July to January in southern Peru. Most of those dissected were wild specimens but a few captive and banded-released individuals were included.

Males come into breeding condition at any season when they reach about two pounds (approximately 7 months of age) and they remain fertile for life. Testes grow rapidly between 14 and 22 mm; all longer than 18 mm contain sperm. The copious vesicular fluid coagulates to form a large vaginal plug after copulation. Females may breed at two pounds if they reach this size at the beginning of the principal breeding season late in October or in November, but do not breed when this small if they reach two pounds during the non-breeding season in August, September, and early October.

Ovulation is near the time of copulation and almost always from the right ovary. Only one ovum is released and it is implanted almost always in the right horn of the uterus. Both ovaries seem to have equal potentialities until a few weeks before estrus when the right ovary becomes larger than the left and contains more medium and large follicles. The left ovary and uterus function if the right ovary is removed.

Accessory corpora lutea begin to appear in the right ovary after about 6 weeks of pregnancy until there may be 12 corpora at parturition. More than a month is required for the embryo to reach the primitive streak stage, and probably over two months more to reach the half-pound birth weight.

Post-parturitional estrus and pregnancy occur occasionally. Lactation lasts more than a month, but the young probably do not require milk. Not more than three young are produced annually by each adult female.

LITERATURE CITED

- ANDREWS, F. N., AND F. F. MCKENZIE 1941 Estrus, ovulation, and related phenomena in the mare. Missouri Agric. Exp. Sta., Research Bull., 329: 1-117.
- ARAI, H. 1920 On the postnatal development of the ovary (albino rat), with special reference to the number of ova. Am. J. Anat., 27: 405-462.
- ASDELL, S. A. 1946 Patterns of Mammalian Reproduction. Comstock Publ. Co., Ithaca. x + 437 pp.
- BAKER, J. R., AND T. F. BIRD 1936 The seasons in a tropical rain-forest (New Hebrides). Part 4. Insectivorous bats (Vespertilionidae and Rhinolophidae). J. Linn. Soc. London (Zool.), 40: 143-161.
- BRAMBELL, F. W. R. 1935 Reproduction in the common shrew (*Sorex araneus* Linnaeus). Phil. Trans. Roy. Soc. London, B, 225: 1-62.
- CAMUS, L., AND E. GLEY 1922 Action coagulante du liquide prostatique de la viscacha sur le contenu des vésicules séminales. C. R. Soc. Biol. Paris, 1922 (2): 207.
- CIESLAK, E. S. 1945 Relations between the reproductive cycle and the pituitary gland in the snake *Thamnophis radix*. Physiol. Zool., 18: 299-329.
- COLE, H. II., C. E. HOWELL AND G. H. HART 1931 The changes occurring in the ovary of the mare during pregnancy. Anat. Rec., 49: 199-209.
- COURRIER, R. 1927 Étude sur le déterminisme des caractères sexuels secondaires chez quelques mammifères à activité testiculaire périodique. Arch. de Biol., 37: 173-334.
- DEANESLY, R., AND T. WARWICK 1939 Observations on pregnancy in the common bat (*Pipistrellus pipistrellus*). Proc. Zool. Soc. London, A, 109: 57-60.
- DENGLER, J. 1940 Contribuciones al estudio de la chinchilla. Las épocas del celo y de las pariciones. An. Soc. Cient. Argent., Buenos Aires, 130: 129-136.
- DUVAL, M. 1895 Études sur l'embryologie des chéiroptères. J. Anat. et Physiol., 31: 93-160.
- ENDERS, R. K., O. P. PEARSON AND A. K. PEARSON 1946 Certain aspects of reproduction in the fur seal. Anat. Rec., 94: 213-227.
- ENGLE, E. T. 1926 A morphological and experimental study of the proximal lobes of the prostate of the guinea-pig, *Cavia cobaya*. Anat. Rec., 34: 75-90.
- EVANS, H. M., AND O. SWEZY 1931 Oogenesis and the normal follicular cycle in adult mammalia. Mem. Univ. Calif., 9: 116-224.
- FLYNN, T. T. 1930 The uterine cycle of pregnancy and pseudo-pregnancy as it is in the diprotodont marsupial *Bettongia cuniculus*. Proc. Linn. Soc., New South Wales, 55: 506-531.
- FLYNN, T. T., AND J. P. HILL 1939 The development of the Monotremata. Part IV. Growth of the ovarian ovum, maturation, fertilisation, and early cleavage. Trans. Zool. Soc. London, 24: 445-622.
- HAMMOND, J., AND F. H. A. MARSHALL 1925 Reproduction in the Rabbit. Oliver and Boyd, Edinburgh. xxv + 210 pp.

- HARTMAN, C. G. 1932 Studies in the reproduction of the monkey *Macacus* (*Pithecius*) *rhesus*, with special reference to menstruation and pregnancy. *Contrib. to Embryol., Carnegie Inst. Wash.*, 23 (no. 433): 1-161.
- HENNING, W. L. 1939 Prenatal and postnatal sex ratio in sheep. *J. Agric. Res.*, 58: 565-580.
- IBSEN, H. L. 1928 Prenatal growth in guinea-pigs with special reference to environmental factors affecting weight at birth. *J. Exp. Zool.*, 51: 51-94.
- KINNEY, P. B. 1923 Internal migration of the ovum in the guinea-pig. *Anat. Rec.*, 25: 137-138.
- MATTHEWS, L. H. 1937 The female sexual cycle in the British horse-shoe bats, *Rhinolophus ferrum-equinum insulanus* Barrett-Hamilton and *R. hipposideros minutus* Montagu. *Trans. Zool. Soc. London*, 23: 224-267.
- 1939 Reproduction in the spotted hyaena, *Crocuta crocuta* (Erxleben). *Phil. Trans. Roy. Soc. London, B*, 230: 1-78.
- 1941 [1942] Notes on the genitalia and reproduction of some African bats. *Proc. Zool. Soc. London, B*, 111: 289-346.
- REECE, R. P., AND C. W. TURNER 1938 The functional activity of the right and left bovine ovary. *J. Dairy Science*, 21: 37-39.
- SHERMAN, H. B. 1937 Breeding habits of the free-tailed bat. *J. Mammal.*, 18: 176-187.
- SWEZY, O., AND H. M. EVANS 1930 Ovarian changes during pregnancy in the rat. *Science*, 71: 46.
- WARWICK, B. L. 1926 Intra-uterine migration of ova in the sow. *Anat. Rec.*, 33: 29-33.
- WIMSATT, W. A. 1944 Growth of the ovarian follicle and ovulation in *Myotis lucifugus lucifugus*. *Am. J. Anat.*, 74: 129-173.

PLATE 1

EXPLANATION OF FIGURES

4 An adult female viscacha with the fur parted over the left nipple to show its unusual location.

5 Uteri and ovaries of a viscacha on about the 32nd day of pregnancy. The uterine swelling before being opened was 14 mm wide and 18 mm long. The embryo is a filmy oval about 2.5 mm long. 2 X.

6 Diagram explaining figure 5.

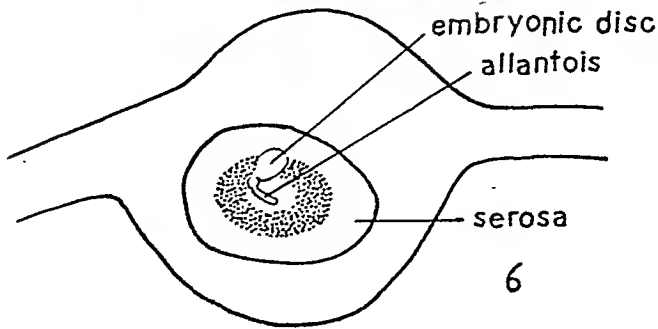


PLATE 3

EXPLANATION OF FIGURES

A comparison of the right and left ovaries of viscachas at different times in the reproductive cycle. The ovaries are sectioned in an antero-posterior plane, and the plane of greatest development of each ovary is shown. The right ovary is mounted at the right in each pair. All 16 X.

10 Ovaries of an older viscacha than the one represented in figure 9. A pro-estrous, possibly parous individual. The left ovary contains only a thin cortex of ova and tiny follicles.

11 Ovaries of a parous, early-pregnant female. Embryo not yet visible macroscopically in the uterus. A large corpus luteum is forming in the right ovary. Follicular development at this time is almost completely suppressed in both ovaries.

12 Ovaries of a parous female with a 29-mm embryo in the right uterus. The right ovary contains the primary corpus luteum, several accessory corpora, and one luteinizing follicle (at 4 o'clock) with a large antrum and retained ovum. One polar body has been given off by this ovum and the remaining chromosomes are at metaphase. Both ovaries contain follicles of full pre-ovulatory size.

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THE ROLE OF MACROPHAGE MOVEMENTS IN THE TRANSPORT AND ELIMINATION OF INTRA- VENOUS THORIUM DIOXIDE IN MICE¹

THOMAS W. EASTON²

Arnold Biological Laboratory, Brown University, Providence, R. I.

SIX FIGURES

INTRODUCTION

Although the occasional appearance of macrophages in the circulation is well known, some authors have indicated that macrophages travel in the blood with something more than occasional frequency as in the splenic veins (Siebel, 1886; Hoffman and Langerhans, 1869), or from the lungs to the liver (Foot, '23), or from various organs into the lungs (Lambin, '32; Irwin, '32). Incidental observations made in this laboratory on mice injected with Thorotrast followed by carbon tetrachloride suggested that large numbers of macrophages move through the liver (Wilson and Leduc, '50; Wilson, Leduc and Corner, '50; Wilson, '51). The investigations here reported were undertaken to determine whether macrophages do move in the blood in significant numbers, and, if so, in what pattern.

The problem has been attacked by combining histological observations with measurements of the quantity of thorium dioxide in various parts of the body in mice, and, by a summation, in the whole body. Since thorium is naturally radio-

¹This paper embodies the essential elements of a thesis submitted in partial fulfillment of the requirements for the Ph.D. degree in June, 1951, at Brown University. The work was supported in part by a grant from the National Cancer Institute, U. S. Public Health Service, administered by Dr. J. Walter Wilson. The guidance and assistance of Dr. Wilson are most gratefully acknowledged. Thanks are also due the Heyden Chemical Corp. for kindness in providing the Thorotrast used in this investigation.

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active, the measurements of amounts of thorium dioxide have been made by measuring the radioactivity of each organ or part, in a manner similar to that employed by Maxfield and Mortensen ('41) in determining the rate of removal of ThO_2 from the blood.

The results obtained indicate that thorium-laden macrophages move into the liver from other parts. They are temporarily held in the liver, and then go into the lungs in the circulation, where they may be eliminated through the respiratory passages. Small amounts of ThO_2 are excreted in urine and bile.

MATERIALS AND METHODS

Animals used were albino mice from the inbred strains BUA, BUB, BUC, and BUD, maintained at the biological laboratory of Brown University. Unless otherwise stated, they were fed on Purina Laboratory Chow.

The material used for injection was Thorotrast, obtained from the Heyden Chemical Corporation of New York. Thorotrast is a colloidal preparation of thorium dioxide in aqueous medium, approximately 25% by volume ThO_2 , or 19% to 20% by weight. The preparation is stabilized by addition of a dextrin preparation, which is 16% to 19% by weight of the whole. Methyl p-hydroxy benzoate (0.15%) is added as a preservative. This analysis of Thorotrast is as given in the American Medical Association's Council on Pharmacy Report ('32).

The general plan of experiments was as follows: Groups of mice, about 60 days of age at the start of the experiments, and ranging in weight from 25 to 30 gm, were injected in the tail vein with Thorotrast, in doses of 0.2 cm^3 every second day, with different groups of animals receiving total amounts of 0.2, 0.3, 0.4, and 0.6 cm^3 .² If necessary, the final dose was

² Radiographs were made of most of the mice used in these studies on the day after final Thorotrast injection. Since ThO_2 is an oxide of heavy metal, dense deposits show well in x-ray pictures, and provide a means of judging the success of injections. Detailed discussions of these are included in the thesis on which this paper is based. These are not significant for the general study, but brief reference is made to some of the radiographs in the description of results obtained with underfed mice.

modified in quantity to achieve the desired total. In each group, one or two animals were killed for examination on the day following completion of injections, and others at intervals thereafter.

Of the mice injected with Thorotrast, but otherwise untreated, 31 received 0.6 cm^3 , 10 received 0.4 cm^3 , 9 received 0.3 cm^3 , and 8 received 0.2 cm^3 .

Eight mice were splenectomized from 7 to 14 days before administration of Thorotrast, then treated as above.

Two groups were injected subcutaneously with 0.1 cm^3 of a 40% by volume solution of CCl_4 in sesame oil, which is a dose sufficient to cause some central necrosis in the liver. One group of 11 mice received CCl_4 one day after completion of the Thorotrast injections; the other group (of 8 mice) received CCl_4 7 days after the last Thorotrast injection.

Two more groups were placed on restricted food intake. The average daily food intake per mouse was found to be 4 gm. Mice on restricted intake were allowed 2 gm daily, in two feedings of 1 gm each, at 8:00 A.M. and 5:00 P.M., with water ad lib. The food for these mice was pulverized Purina Laboratory Chow. One group of 8 mice was restricted in food intake 14 days before Thorotrast injections were begun; the other group (of 10 mice) was restricted in food intake 7 days after the final Thorotrast injection. Both groups were continued on restricted food intake throughout the experiment.

Four mice were injected with 0.6 cm^3 of Thorotrast and their entire output of urine and feces was collected every second day for measurement of the amount of Thorotrast eliminated by these routes. These mice were kept in small cages with two levels of screening above the cage floor. The upper screen was of $\frac{1}{4}$ -inch mesh, and the lower of 16-mesh window screen. Feces were collected on the second screen, and the urine was absorbed in filter paper cut to fit the bottom of the cage. Feces and urine-soaked papers were assayed for ThO_2 in the same manner as parts of animals.

Before proceeding to investigations of the pattern of Thorotrast disposition in the body of the mouse, an attempt was

made to determine in which parts of the body Thorotrast might be localized, without attention to quantitative aspects. Six mice, previously injected with 0.6 cm^3 of Thorotrast, were killed and samples of various organs and tissues were taken for assay of ThO_2 and for histological examination. Both assayed and histological samples were treated as were the samples taken for quantitative estimates, according to the procedure indicated below.

For histological study, several pieces (less than 1% of the organs) were taken in all experiments of livers, spleens, and lungs from all animals, as well as kidneys from the mice on restricted food intake, and a series of control animals. All were fixed in Bouin's fluid, sectioned in paraffin at 5μ , and stained with Delafield's hematoxylin and eosin.

For measurements of amounts of ThO_2 in the body, mice were killed by gas to avoid loss of blood, and after histological samples had been removed, were divided into fractions as follows: (1) liver, (2) spleen, (3) lungs, (4) other viscera, including heart, thymus, stomach, intestines, pancreas, kidneys, adrenals, and sex organs, with the lymph nodes of the abdominal and thoracic cavities, (5) head and neck, (6) forequarters, and (7) hindquarters and tail. In some of the early work spleen and lungs were taken together as a single fraction.

Fractions thus separated were placed in small aluminum or steel dishes, and were incinerated thoroughly in an electric furnace at 600°C . for at least 18 hours, usually a little longer. After incineration, the dishes were allowed to cool, and the ash was then flattened carefully in the bottom of the dishes with the point of a fine scalpel. Thorough incineration is necessary if the ash of bone is to crumble readily, otherwise particles of bone-ash may fly about when crushing is attempted, causing possibly significant loss of radioactivity.

Measurements of radioactivity were made with a Tracerlab SC 1-b Autoscaler and Geiger tubes of 1.6 and 1.9 mgm/cm^2 window thickness. Samples of Thorotrast were tested individually, and, although each sample differed slightly in activity

from the others, each was found to yield a constant number of counts per minute per unit volume, after ashing. By giving mice injections from a single bottle of Thorotrast only, it was thus possible to equate counts per minute with definite volumes of Thorotrast. During actual counting, background was checked once per hour, at least, and was found constant at 35 counts per minute. At least once per day of counting, a 22- μ g sample of Bi^{210} was counted as a check on the tube in use. At the beginning of every counting period, the stop-clock used for timing counts was also checked. Experimental material was always counted at 4 times the background rate, at least, usually much higher.

All experimental samples were counted in the same dishes in which they were incinerated, at 1 cm from the tube window. Calculations following Friedlander and Kennedy ('49) indicate that in this position, and under the conditions imposed, all of the radiation of thorium and its decay products, if present, could be counted, with the following exceptions: the beta emission of Ra^{228} (mesothorium₁) would not be counted under any circumstances; about 20% of the beta emission of Pb^{212} would not be counted because of absorption in the ash of the sample and in the tube window; and the gamma radiation of the various members of the decay series would be counted at a low efficiency, which would, however, be constant. Thus, the counts include essentially all of the alpha emission, most of the beta emission, and a low, constant percentage of the gamma emission of the thorium series when all of the members of the series are present in equilibrium. That they are not all present in equilibrium in recently manufactured samples of Thorotrast was indicated by comparison of the number of counts per minute obtained from volumes of a recently made sample and a sample 17.5 years old, obtained from the manufacturers. The older sample yielded about twice the activity of the younger. Since the samples used were not at equilibrium, it is important to know the possibility of significant change in their activity during their period of use. Calculations (again following Friedlander and Kennedy,

'49) showed that the number of counts per minute to be expected from any particular sample of Thorotrast might rise or fall by 5% during a 6-month period within 1.5 and 2.5 years after chemical purification of ThO_2 in the process of its manufacture. Over short periods (up to 50 days) the change would be very slight and could be ignored. Actually the critical quantitative work of these investigations was done in periods of less than 50 days' length. However, tests on different samples of Thorotrast revealed no significant change at intervals of 5 months.

There is thus the possibility of an error of 5% in the counting data, if experiments are continued over 6 months, due to change in the activity of Thorotrast. At shorter times the error becomes very small, and is insignificant in the period of most of these experiments. There is also the possibility of a maximum statistical error of 3% in the counting, plus a small error in measuring volumes of Thorotrast. The results reported are believed accurate to within 5%.

RESULTS

General distribution

Assays of radioactivity of various parts of the body with no attempt to measure quantities gave a general idea of the areas in which intravenous ThO_2 localizes. Skin yielded no activity; skeletal muscle had a low level of activity, so low that it could not be equated with a volume of Thorotrast; and the gut, either taken in sections or in toto, also gave a barely perceptible but non-measurable activity. Kidneys, adrenals, heart, thymus, sex organs, including uterus, and abdominal and thoracic lymph nodes taken as a single sample with the gut yielded measurable activity, but taken alone as single samples, did not. This group of organs is subsumed under "all other viscera" in tables giving activity data below. Brain and spinal cord yielded no activity. Essentially all of the activity was confined to the liver, spleen, lung, and bone.

Histological surveys of the same tissues and organs agreed with the assay data, with one or two exceptions. No ThO_2

could be found in the skin, nor in nervous tissue. Skeletal muscle, thymus, lymph nodes, and kidney revealed widely separated minute deposits in macrophages. In the gut, there were also small, scattered deposits of ThO_2 in macrophages in the wall, but none at all in association with the epithelium, in any part. Massive amounts could be observed in macrophages in livers, spleens, lungs, and red bone marrow (in smears). The exceptions mentioned above are the adrenals, pituitary, and testis or ovary, all of which may contain fairly large numbers of macrophages loaded with ThO_2 . However, the small size of these organs renders them insignificant as depots of ThO_2 , in comparison with the much larger quantities elsewhere in the body.

Intravenously injected Thorotrast is nearly completely removed from the circulation in 4 hours in mice, and is entirely removed in less than 24 hours. Thereafter it is only to be found in macrophages, principally in the liver, spleen, lung, or marrow. Radioactivity assays of weighed bits of these organs (marrow excepted) indicate that within any particular organ counts per minute per unit weight are constant throughout. This indicates that thorium dioxide is regularly distributed in the organs in such fashion that histological sections from any part should represent conditions prevailing throughout.

Radioactivity assays

Retention of injected Thorotrast. Measurements of amounts of ThO_2 retained by mice at various times after injection indicate that significant quantities are retained for very long periods of time. On the first day after injection some of the mice examined retained as little as a third of the total injected dose, while others retained essentially all of it. Four mice assayed on the day after the final injection of Thorotrast to a total of 0.6 cm^3 retained 0.59, 0.58, 0.57, and 0.60 cm^3 , representing an essentially complete retention of the injected material. Two mice, assayed one year after the injection of 0.6 cm^3 of Thorotrast, retained the equivalent of approximately 0.2

cm³. It appears that the period in which injections are made, and the 10 days following completion of injections, constitute the time when the most rapid loss of Thorotrast occurs. The amount retained varies widely in the first 10 days, and thereafter is more nearly constant, in spite of considerable individual variation. Ten of 19 mice (table 2) killed between 11 and 94 days after injection of 0.6 cm³ retained amounts between 0.35 and 0.45 cm³ (average 0.40). The average amounts of ThO₂ retained in groups of mice variously treated are

TABLE 1
Retention of Thorotrast in groups of mice under various conditions

| DOSE CM ³ | OTHER TREATMENT | NUMBER OF MICE | AV. WT. LEFT (GRAMS) | S.E. | STANDARD DEV. |
|-------------------------|--------------------|-------------------|-------------------------|-------|------------------|
| 0.6 | None | 31 | 0.40 | 0.10 | 54 |
| 0.6 | Splenectomy | 8 | 0.38 | 0.043 | 100 |
| 0.6 | RFI-14 | 8 | 0.35 | 0.055 | 20 |
| 0.6 | RFI-7 | 10 | 0.23 | 0.057 | 47 |
| 0.4 | None | 10 | 0.17 | 0.013 | 181 |
| 0.3 | None | 9 | 0.15 | 0.011 | 27 |
| 0.2 | None | 8 | 0.10 | 0.015 | 30 |

RFI-14 = Restriction of food intake 14 days before injection of Thorotrast, and subsequently to the end of experiment.

RFI-7 = Restriction of food intake 7 days after injection, and subsequently to end of experiment.

given in table 1, expressed as equivalents of volumes of Thorotrast.

Unlike the groups of mice described in table 1, those mice treated with CCl₄ in addition to Thorotrast did not lose ThO₂ more noticeably during the first 10 days after injection, but continued to lose it at a more rapid rate throughout the experiments. Eleven mice received CCl₄ on the day after the final Thorotrast injection (0.6 cm³ total), and 8 others, 7 days after injection of the same dose. The last mouse of the former group was killed 42 days after injection, at which time there remained 0.2 cm³ in the body, with all the mice exhibiting a steady decrease in the amount of Thorotrast retained through-

out the experiment. The last mouse of the second group was killed 23 days after the final Thorotrast injection, when 0.3 cm^3 was retained. Mice of this group killed earlier also exhibited a steady decline in amount of Thorotrast retained.

Alterations in distribution. Analysis of the amounts of ThO_2 in the various parts of the bodies of mice of the different experiments indicates that ThO_2 does not remain distributed in the body in the manner in which it is first taken up. If, for each mouse, the percentage of the ThO_2 in the body is calculated for each organ or part, it is evident that the liver comes to contain progressively more of the total ThO_2 , at the expense of the rest of the body, and of the skeleton (red bone marrow), particularly. It seems justified to ascribe the activity observed in parts containing skeletal material to the marrow, since assays of skeletal muscle and skin with their associated connective tissue reveal extremely little activity, and histological observations confirm the results of assays in that ThO_2 appears in such tissues only as very small deposits, widely separated.

Tables 2 and 3 present the percentages of ThO_2 in various parts of the body for two groups of mice injected with Thorotrast, but otherwise untreated. Both groups exhibit an increase in the fraction of the total ThO_2 held by the liver.

The same increase in the percentage of the total ThO_2 held in the liver occurs in splenectomized mice and in mice treated with CCl_4 . The increase is slower in splenectomized mice, since the percentage of ThO_2 is still increasing at 100 days after injection, at which time the liver contains 84% of the ThO_2 in the body. In mice injected with CCl_4 the increase in the percentage of total ThO_2 in the liver is paralleled by an unusually rapid loss of ThO_2 from the body.

In mice on restricted food intake there is no steady increase in the percentage of the ThO_2 in the liver. Table 4 presents the data for a group of mice on restricted food intake compared with a similar group injected with the same dose (0.6 cm^3) of Thorotrast, but otherwise untreated. Although the percentages retained in the organs of the experimental mice of table 4 vary, they do not show the trends of

increasing or decreasing percentages of the "normal" mice. Apparently the restriction of food intake results in a greater variability in the pattern of Thorotrast retention and loss.

Percentages of ThO_2 in the various parts of mice injected with 0.3 and 0.4 cm^3 of Thorotrast, and with CCl_4 in addition

TABLE 2

Percentages of total Thorotrast retained in various organs and parts of 23 mice injected with 0.6 cm^3

| KILLED DAYS AFTER INJECTION | PERCENTAGES RETAINED IN | | | | TOTAL CM^3 IN BODY AT SACRIFICE |
|-----------------------------------|-------------------------|--------------------|----------------------|----------|--|
| | Liver | Spleen and lung | All other viscera | Skeleton | |
| 1 | 46.0 | 20.3 | 12.9 | 20.8 | 0.54 |
| 3 | 59.0 | 13.1 | 4.2 | 23.6 | 0.38 |
| 4 | 63.5 | 17.4 | 2.4 | 16.7 | 0.49 |
| 7 | 47.7 | 15.8 | 2.9 | 33.6 | 0.38 |
| 11 | 46.6 | 33.6 | 2.8 | 17.1 | 0.39 |
| 14 | 65.6 | 22.5 | 5.0 | 10.3 | 0.53 |
| 17 | 62.8 | 24.6 | 2.2 | 10.3 | 0.45 |
| 20 | 54.2 | 28.6 | 2.6 | 14.6 | 0.35 |
| 23 | 58.2 | 19.9 | 2.6 | 19.3 | 0.30 |
| 26 | 53.3 | 18.6 | 3.1 | 25.0 | 0.29 |
| 29 | 72.4 | 16.2 | 1.9 | 9.7 | 0.46 |
| 32 | 71.0 | 16.7 | 1.4 | 10.7 | 0.48 |
| 35 | 69.0 | 21.1 | 1.3 | 8.5 | 0.52 |
| 38 | 80.0 | 5.4 | 3.2 | 11.5 | 0.41 |
| 45 | 49.0 | 19.1 | 4.6 | 27.3 | 0.28 |
| 52 | 47.2 | 17.1 | 6.5 | 29.2 | 0.26 |
| 59 | 54.0 | 26.1 | 2.2 | 17.6 | 0.45 |
| 66 | 61.3 | 23.5 | 2.2 | 12.6 | 0.46 |
| 73 | 57.5 | 26.0 | 3.3 | 12.8 | 0.36 |
| 80 | 61.6 | 24.2 | 3.0 | 11.2 | 0.43 |
| 87 | 68.5 | 21.6 | 1.8 | 7.9 | 0.49 |
| 94 | 72.4 | 16.4 | 2.5 | 8.7 | 0.44 |
| 94 | 79.9 | 3.5 | 3.3 | 13.4 | 0.37 |

to Thorotrast, are nearly identical with the percentages given for mice injected with 0.6 and 0.2 cm^3 in tables 2 and 3.

Histological observations

Mice injected only with Thorotrast. Histological observations provide evidence of macrophage movement in the same

nuclei of a given blastema have only one nucleolus, which is large and obviously the result of coalescence (fig. 14). Furthermore, when this phenomenon of generalized coalescence of nucleoli occurs in one limb, it is always present in the opposite limb. There were no examples of the phenomenon in groups 1 b and 2 and further work is required to clarify this interesting coalescence. In stage 4 when proliferation is the dominant process the blastema cells tend to have the maximum number of nucleoli, whether it be two or three (figs. 15, 19).

The phenomenon of coalescence of nucleoli has been studied in plants by Kostoff ('47). Measurements show that the nucleolus resulting from fusion of separate nucleoli in polyploid cells has a volume proportional to the number of nucleoli composing it. No similar measurements were made in the present study, but on inspection this appears to be approximately so for the tadpole cells. Single nucleoli are of fairly uniform size (figs. 15, 17, 18, 19) whereas a nucleolus composed of fused nucleoli is considerably larger (fig. 14). However, because size measurements of nucleoli present greater problems than measurements of nuclei, it is not possible in this series to judge degree of polyploidy from fused nucleoli as Kostoff does on plants. The generalized coalescence of nucleoli in stages 2 and 3 of group 1 a is described in the preceding paragraph. Determination of chromosome number by nucleoli was thus impossible in the majority of the blastemas in these crucial stages in group 1 a.

Because haploid cells have but one nucleolus which can be confused with the coalesced nucleolus of diploids or triploids, it is not possible to judge chromosome number by nucleoli alone in isolated haploid nuclei. Nuclear size together with the small nucleolus can identify haploid epithelium in mass (fig. 17). However, size variations are so great in the blastema that it is impossible to identify isolated haploid cells there by these criteria. Therefore, in group 2 (haploid transplants) the column in table 1 for blastema transplant cells is left blank.

C. Inflammatory reaction to the transplant

Figure 16 is a low power photograph of a typical inflammatory reaction to the transplant. Occurring always in the proximal part of the limb, this reaction involves an accumulation of mononuclear leucocytes predominantly, a few polymorphonuclear leucocytes, edema and necrosis. The phagocytes actually invade the transplanted epidermis in some cases (fig. 16). However, transplanted epidermis which has migrated distally to the area of regeneration is not invaded by phagocytes.

The inflammatory reaction to the transplant is at a peak in stages 2 and 3, fading out in stage 4 (see table 1). This may or may not be related to the disappearance of detectable transplant cells. Inflammatory reactions are not as frequent or as extensive in the control limbs (table 1). It must be concluded that the tadpole limb is capable of reacting to foreign tissue at least by inflammation.

D. Behavior of the transplanted basement membrane

The basement membrane of the transplanted epidermis is thick (figs. 3, 4). As illustrations 4 and 5 in figure 1 demonstrate, this basement membrane does not migrate with the epidermis but is left behind and marks the original location of the transplanted epidermis. In figures 6 and 7, this basement membrane can be seen at the base of the limb. It is no longer covered with transplanted epidermis. When the host's own basement membrane regenerates, this landmark can no longer be distinguished. In stage 5, therefore, it is impossible to delineate the transplant's basement membrane and it is not known how long this noncellular material remains on the host limb.

E. Fate of the transplanted epidermal cells

Individual observations are listed in table 1. In general, the results are as follows.

Stage 1. The epidermal transplant (fig. 3) was placed on the thigh of the tadpole's hindlimb (fig. 4). These epidermal cells migrate to the tip of the limb (fig. 5), tending to remain in a coherent sheet, so that patches of solid transplant epithelium can be found later both on the proximal limb (fig. 17) and at the tip. This is accomplished without mitosis (Herrick, '32; Arey, '32). The epithelium moves over a

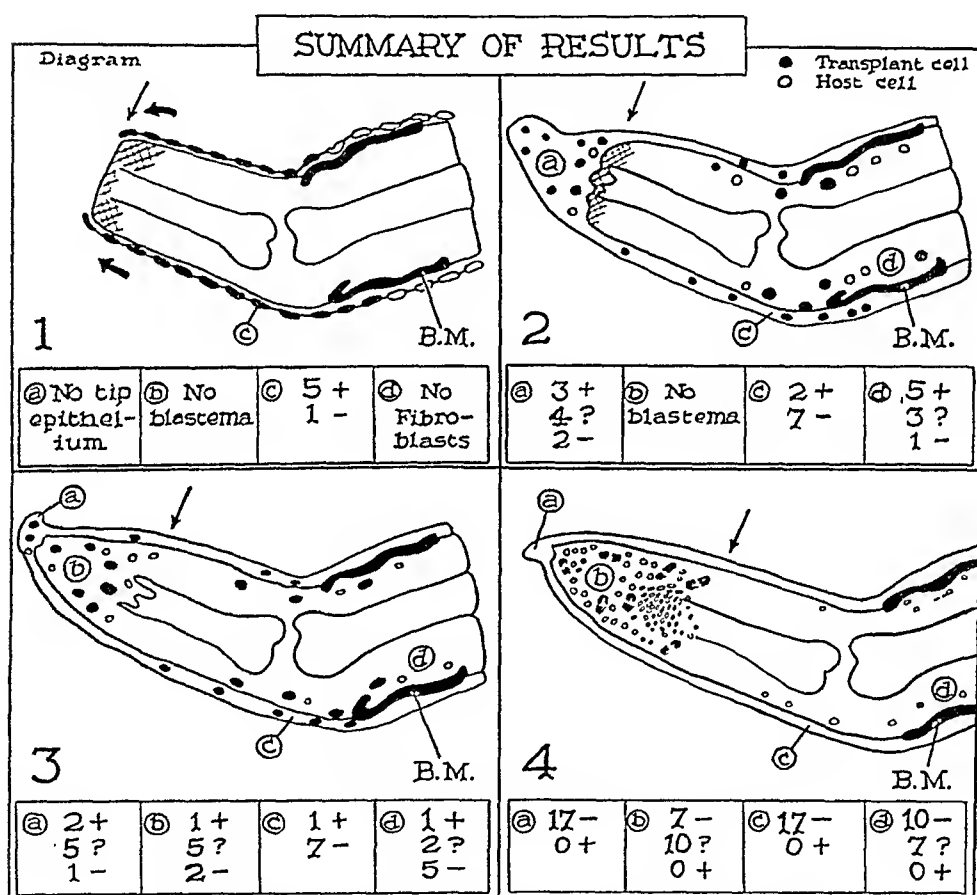


Fig. 2 Diagram summarizing the experimental results.

(1) Stage 1, epithelial migration. Thin arrow indicates amputation level; thick arrows show direction of migration. B.M. is the basement membrane of the transplant. (2) Epithelial mound stage. (3) Early blastema stage. (4) Stage of proliferation and differentiation.

The positive and negative results are listed as numbers of limbs in the columns at the bottom of each drawing. These columns represent the 4 regions in which transplant cells are found: (a) Tip epithelium; (b) blastema; (c) epithelium on side of limb; (d) fibroblasts at base of limb. Transplant cells are indicated by black dots.

fibrin surface (stained black in fig. 5) similar to that described by Brown ('39). As indicated in figure 2 (1), there is no tip epithelium or blastema in this stage.

Stage 2. In the epidermal mound which now forms at the tip, significant numbers of transplanted epidermal cells were found in three cases. Of the other 5 cases in this stage, two had no discoverable tip epidermis from the transplant and three were questionable cases (see table 1). A few cells apparently not derived from the transplant are now present in the blastema.

Many of the limbs which received a triploid epidermal transplant have triploid fibroblasts in the proximal limb in this stage. It seems likely that these fibroblasts arose from the transplanted epidermis for the following reasons: (1) no such fibroblasts were seen in stage 1 before the transplanted epidermis began to disappear; (2) the transplant carries over no significant amount of subepithelial tissue (fig. 3) and very little proliferation occurs before stage 4, so that epithelium is the only tissue present in sufficient amount to furnish the numerous triploid fibroblasts found; (3) the transplant is usually unavoidably curled back on itself in many regions when placed on the limb, which locates many of the epithelial cells internally where they appear to transform into fibroblast-like cells (figs. 6, 7, 8).

Stage 3. By this time the chromatin of the wound-epithelial cells stains so darkly that most of the nucleoli are obscured (fig. 20). This prevents accurate identification of triploid cells. The two limbs in this stage which received haploid transplants had identifiable haploid epidermis in the epidermal mound at the tip of the limb.

In this stage, which is characterized by a receding mound of epidermis and a medium to large blastema without appreciable proliferation, one of the 4 limbs in group 1a contained transplant cells in the blastema. This is Hg 21 in table 1. This limb received an epidermal transplant that was probably completely tetraploid, although this cannot be judged from the nucleoli alone since some cells have 3 and others 4 nu-

cleoli (see above section on nucleoli). In the blastema of this limb, there are numerous cells with 3 and 4 nucleoli as illustrated by three representative sections in figures 20, 21 and 22. The control limb, which received no transplant, is completely diploid as expected (fig. 20). As can be seen by comparing figure 19 with figures 20, 21 and 22, the blastema cells originating from the polyploid epidermal transplant are considerably larger than those of the control limb. (However, this size difference is exaggerated here for the blastema of the control limb is proliferating.) The morphology of the transformed polyploid epidermal cell is characteristic of the mesenchyme-like early blastema cell with relatively clear nucleus and diffuse cytoplasm (fig. 21). Many of the blastema cells are smaller, possibly diploid.

The question arises as to whether this early regeneration blastema is composed entirely of cells from the epidermis, but this cannot be answered from the present material. In the first place, nucleoli will not identify all the transplant cells therein because many of them will be fused and there is no way of estimating how many polyploid cells with fused nucleoli are present. Cell size cannot be used accurately, more especially here than in any other stage, because of enormous variation together with the lack of control tetraploids. In the second place, it is not reasonable to expect, on the basis of the results in the other limbs, that the entire epithelial cap was composed of transplant cells. In addition to host internal tissues, some host epidermis was undoubtedly in a position to contribute to the blastema.

A second question which arises is whether these polyploid blastema cells might have come from subepithelial tissue carried over with the epidermal transplant (fig. 3). Measures were taken to guard against this possibility in the way the operation was planned. Because epithelium is known to migrate to cover a denuded surface, the transplant was placed on the proximal limb a distance from the amputation. The basement membrane of the transplanted epidermis remains on the thigh (figs. 1, 6, 7) when the epidermis migrates to

the amputation surface distally. It seems likely that any small amount of subepithelial tissue carried over remains with this basement membrane. Because the blastema presumably forms locally (Butler and O'Brien, '42), such tissue will not be in a position to contribute to the regeneration blastema. Furthermore, the point noted above in connection with triploid fibroblasts applies here, namely, that the transplant is primarily epidermis (figs. 3, 4) and almost no mitosis occurs in stages 1-3. Therefore, the polyploid epidermis is the only tissue present in sufficient amount to furnish the very numerous polyploid blastema cells found.

Finally, the significance of only one positive result may be questioned. In the first place, the advantage of this method is that it is qualitative. We found that nucleoli, carefully defined, could be relied upon as an index of polyploidy. The triploid hosts in group 2 were always identifiable (figs. 15, 18). There were so many large blastema cells with 3 and 4 nucleoli in this experimental limb that the presence of the transformed polyploid transplant inside the blastema is hardly questionable.

In the second place, a consideration of the remaining limbs in this stage reveals that there were but two actual negative results. These were in group 1 b, which was the preliminary experiment with poorer technique and is noteworthy for its general lack of positive results. In group 1 a, there are three limbs in stage 3 besides the positive result reported above. However, these three have fused nucleoli in the blastema cells and cannot be identified, as was pointed out in the section on nucleoli. None of the blastemas in group 2 can be judged because the transplant was haploid and isolated haploid cells are not recognizable in the growing blastema. Under these conditions, it is not unreasonable to have only one positive blastema.

Stage 4. This stage, beginning 7 to 8 days after amputation and characterized by proliferation and a certain amount of differentiation, shows a notable lack of transplant cells. It is possible that some of the transplanted cells have dis-

appeared into differentiating tissue, but there are present so many proliferating blastema cells easily identified by nucleoli that this is not a reasonable explanation for the complete disappearance of the transplant. There are no longer any triploid fibroblasts at the base of these limbs, and no more triploid or haploid epithelium can be found. It seems that something has happened in stage 4 to eliminate all the transplanted cells. As can be seen in table 1, the inflammatory reaction to the transplant now fades out, yet this was present, with two exceptions, in every case with detectable transplant cells in stages 2 and 3. Perhaps the immune reactions of the host have overcome the transplant tissue. If this is the case, it is remarkable that this did not occur until stage 4 and then occurred without exception, regardless of the age in days of the limb. Haploid tails lived over a month on younger diploid tadpoles (see Materials and Methods). It may be that the lost cells died in some unknown role pertinent to this stage of regeneration. Another possibility is that chromosome number is regulated to diploid in this stage of proliferation. It is not possible to answer these questions from the present material.

DISCUSSION

Both the method of tracing transplanted cells and the results of the cell tracing require some further discussion.

A. The use of heteroploid transplants in cell tracing

The limitations of the use of nuclear size have been pointed out in the section on the identification of the transplanted cells. Several criticisms can be made of Hertwig's work ('27) with haploid transplants in regeneration. Although he uses nuclear size (measured only by two perpendicular diameters of the nucleus) as the only criterion of chromosome number, he does not mention how many cells he measured, what the variation in individual nuclei was, or what the variation in samples of nuclei from different individuals was. The series

he reports consists of three cases of regeneration and 5 of embryonic development. Although his conclusions regarding cartilage, which is a fairly homogeneous cell mass as to cell size, may be valid, his results cannot be evaluated statistically. But he also compares isolated nuclei in the growing blastema to draw conclusions which are not valid. His work is widely quoted as evidence that the blastema arises *in situ* in spite of the fact that his method, as he presents it, is not reliable. By way of comparison with Hertwig, the work of Raven ('37), which considers the statistical side of the problem, should be cited. He reports the use of transplants between two salamander species with different sized cells to determine the embryological origin of sheath cells and sympathetic neuroblasts. Frequency distribution curves of nuclear size together with further statistical analysis led him to a conclusion similar to ours, that compact cell masses of the two species could be distinguished from one another by nuclear size measurements, but that isolated cells cannot be adequately identified by this method because of the overlap in nuclear size.

Because of the limitations of nuclear size as a method of identifying cells, we decided to use nucleoli for the primary criterion. This was a satisfactory method for epithelial cells and cells of the blastema. However, as discussed in the results, we did not put complete reliance on this method when identifying differentiating cartilage and muscle in our preparations. Another limitation of the use of nucleoli in cell identification is the phenomenon of coalescence, which is described in the section on the behavior of nucleoli in regeneration. Since only the maximum number of nucleoli reflects the chromosome number, fusion of nucleoli removes the identifying characteristic.

Certainly some of the limitations of nucleoli in cell identification can be overcome by further work. The staining problem was investigated in the present work, but since all material had been fixed in Bouin's, various fixatives could not be tried. The primary problem in staining the internal tis-

sues mentioned above is chromatin, which tends to imitate nucleoli when clumped. But Bouin's is known to clump chromatin; a more benign fixative should give better results.

Another improvement in the use of heteroploid material in cell tracing would be the use of tissues differing more widely in chromosome number. Faulkhauser and Humphrey ('50) report the production of tetraploids, pentaploids, and heptaploids from triploid axolotl females mated with diploid males. With such large differences in nuclear volume, nuclear measurements could probably be used for cell identification with greater ease.

Finally, it should be pointed out that the use of heteroploid material in cell tracing could be of use in the solution of many embryological problems of cell origin. The advantages of the method are that the criteria for identification are inherent in the cell, the method can be used to trace individual cells, transplants can be made between animals of the same species, and, finally, work being done now on polyploidy in amphibians promises to give information with which the method can be improved.

B. Results of tracing the epithelium

To reiterate, the results of this experiment were that transplanted epidermis was found to migrate to the amputation surface of the limb and, in a certain number of cases, to compose a part of the epithelial mound which forms over the wound. It was possible to demonstrate these transformed epithelial cells inside the early blastema in the next stage of regeneration. But after proliferation begins (stage 4), the transplant can no longer be identified in the limb.

The purpose of this experiment was to test the theory first suggested by Godlewski ('28) that epidermal cells enter the blastema. This experiment has shown that epidermis can undergo transformation into cells in the early blastema. However, two parts of the theory as stated by Rose ('48) are not tested by this experiment and deserve further discussion: (1)

that these transformed epidermal cells are truly dedifferentiated and (2) that they form a major part of the blastema.

It was not demonstrated that epidermis is truly dedifferentiated because the definition of dedifferentiation implies that such cells are capable of redifferentiating into any tissue. Redifferentiation could not be shown in this experiment for reasons discussed in the Results. The transformed cells have the histological characteristics of dedifferentiated mesenchyme-like cells. It is just as valid to conclude from that observation that they are dedifferentiated as it is to judge from histological preparations that the internal tissues dedifferentiate (Thornton, '38). The latter idea has been accepted by many investigators, although it has never been proved by cell tracing. The primary objection to the idea that epidermis contributes cells to the blastema which can redifferentiate into limb tissues is the germ-layer theory. This theory has been questioned many times, recently by Oppenheimer ('40). It does not hold in the regeneration of many invertebrates, where ectoderm can form so-called mesodermal structures (Morgan, '04; Schotte, '40), and exceptions to it exist in amphibian ontogeny, such as the formation of cartilage from the neural crest (Landaere and Warren, '18). Further work is necessary to show conclusively that either ectodermal or mesodermal tissues truly dedifferentiate in amphibian regeneration. The present experiment, together with Rose's observations ('48), make it seem quite possible that epidermis can dedifferentiate.

The second consideration is whether the epidermis forms a major part of the regeneration blastema. This cannot be determined from the present experiment because no attempt was made to give the limb a continual supply of transplant epidermis or to prevent host epidermis from entering into the process. It is an important point because, if the epidermis does supply the majority of cells in the early blastema, Godlewski's skin seals ('28, see Introduction) probably inhibit regeneration by preventing the formation of the blastema from the epidermis. Recently Thornton ('49) came to the

conclusion that the major effect of beryllium in inhibiting urodele limb regeneration seemed to be rapid formation of a thick skin covering at the tip. In these cases, normal regeneration may be inhibited by failure of the epidermis to enter the blastema. But if this is true, epidermis must form the major portion of the early blastema, or else it plays some essential role in initiating dedifferentiation of the other tissues, for otherwise the internal tissues should form some sort of a regeneration blastema.

The literature on the role of epithelium in amphibian regeneration has recently been thoroughly reviewed and discussed by Rose ('44, '45, '48). Since that time, Karczmar and Berg ('51) have studied the distribution of alkaline phosphatase in regenerating larval urodele limbs. These authors state that cells about to become blastema cells can be identified by increased enzyme activity and that, because epidermis did not stain consistently, "No support could be found in phosphatase staining for Rose's contention." However, because they have no criterion for determining what cells become blastema cells other than enzyme activity and enzyme activity has not been proved to be related to the transformation of cells into blastema cells, it is difficult to see how their work is at all relevant to the subject of the origin of the blastema.

In conclusion, it is suggested that no mere mechanical role can be assigned to the epidermis in regeneration, but that the wound epidermis must be regarded as a vital and indispensable part of the process, in which the first step is apparently its transformation into mesenchyme-like blastema cells.

SUMMARY

1. The purpose of the present experiment was to test the theory first suggested by Godlewski and revived and restated by Rose that the wound epidermis of amputated amphibian limbs dedifferentiates to supply a portion of the cells of the young regeneration blastema. To trace the epidermis haploid

and triploid tail epidermis was transplanted to regenerating limbs of diploid and triploid *Rana pipiens* tadpoles.

2. The most reliable criterion for the identification of the transplanted cells proved to be the maximum number of nucleoli in the nucleus, which varies directly with the number of sets of chromosomes. The behavior of nucleoli in regenerating limbs is described and the advantages and limitations of the method are discussed.

3. In the first stage of regeneration, the transplanted epidermis migrates distally toward the amputation surface of the limb. Its basement membrane is left behind on the thigh. In the second stage of regeneration, transplanted epidermal cells may be found in the thick epithelial mound forming at the tip of the limb.

4. In the third stage of regeneration, when the epithelial mound disappears and the early blastema appears, one limb shows numerous large mesenchyme-like triploid and tetraploid cells inside the blastema which have come from the triploid-tetraploid epidermal transplant which this diploid limb received.

5. In stages 4 and 5, proliferation and differentiation begin. The transplanted cells are not present in the epithelium or in the undifferentiated part of the blastema of any of the limbs in these stages. In the differentiating internal tissues it was not possible to identify transplanted cells by the present method because the nucleoli could not be recognized with clarity. Thus, it is not known whether or not the transformed epidermal cells in the blastema can redifferentiate into the cartilage and muscle of the new limb.

LITERATURE CITED

- AREY, L. B. 1932 Certain basic principles of wound healing. *Anat. Rec.*, 51: 299-313.
- BRIGGS, R. 1947 The experimental production and development of triploid frog embryos. *J. Exp. Zool.*, 106: 237-266.
- BROWN, M. W. 1939 The migratory behavior of frog epidermis with special reference to surface polarity and to factors determining direction of movement. *J. Exp. Zool.*, 81: 91-126.

- BUTLER, E. G., AND J. P. O'BRIEN 1942 Effects of localized x-radiation on regeneration of the urodele limb. *Anat. Rec.*, 84: 407-413.
- FANKHAUSER, G. 1945 The effect of changes in chromosome number on amphibian development. *Quart. Rev. Biol.*, 20: 20-78.
- FANKHAUSER, G., AND R. R. HUMPHREY 1943 The relation between number of nucleoli and number of chromosome sets in animal cells. *Proc. Nat. Acad. Sci. Wash.*, 29: 344-350.
- 1950 Chromosome number and development of progeny of triploid axolotl females mated with diploid males. *J. Exp. Zool.*, 115: 207-250.
- FORSYTH, J. W. 1946 The histology of anuran limb regeneration. *J. Morph.*, 79: 287-321.
- GIDGE, N. M., AND S. M. ROSE 1944 The role of larval skin in promoting limb regeneration in adult Anura. *J. Exp. Zool.*, 97: 71-93.
- GODLEWSKI, E. 1928 Untersuchungen über Auslösung und Hemmung der Regeneration beim Axolotl. *Arch. für Entw.-mech.*, 114: 108-143.
- HERRICK, E. H. 1932 Mechanism of movement of epidermis, especially its melanophores, in wound healing, and behavior of skin grafts in frog tadpoles. *Biol. Bull.*, 63: 271-286.
- HERTWIG, G. 1927 Beiträge zum Determinations- und Regenerationsproblem mittels der Transplantation haploidkerniger Zellen. *Arch. für Entw.-mech.*, 111: 292-316.
- KARCZMAR, A. G., AND G. G. BERG 1951 Alkaline phosphatase during limb development and regeneration of *Amblystoma opacum* and *Amblystoma punctatum*. *J. Exp. Zool.*, 117: 139-164.
- KOSTOFF, D. 1949 Concerning the number and size of nucleoli. *Proc. 6th Int. Cong. Exp. Cytol.*, Stockholm. Suppl. 1: 134-136.
- LANDACRE, F. L., AND J. H. WARREN 1918 The origin of cartilage from ectoderm in the urodeles. *Anat. Rec.*, 14: 42-43.
- MORGAN, T. H. 1904 Germ layers and regeneration. *Arch. für Entw.-mech.*, 18: 261-264.
- OPPENHEIMER, J. H. 1940 The non-specificity of the germ layers. *Quart. Rev. Biol.*, 15: 1-27.
- RAVEN, C. P. 1937 Experiments on the origin of the sheath cells and sympathetic neuroblasts in amphibia. *J. Comp. Neur.*, 67: 221-240.
- ROSE, S. M. 1944 Methods of initiating limb regeneration in adult Anura. *J. Exp. Zool.*, 95: 149-170.
- 1945 The effect of NaCl in stimulating regeneration of limbs of frogs. *J. Morph.*, 77: 119-139.
- 1948 Epidermal dedifferentiation during blastema formation in regenerating limbs of *Triturus viridescens*. *J. Exp. Zool.*, 108: 337-361.
- SCHOTTE, O. E. 1940 The origin and morphogenetic potencies of regenerates. *Growth*, Suppl., 59-76.
- SCHOTTE, O. E., AND M. HARLAND 1943 Amputation level and regeneration in limbs of late *Rana clamitans* tadpoles. *J. Morph.*, 73: 329-363.

- THORNTON, C. S. 1938 The histogenesis of the regenerating forelimb of larval *Amblystoma* after exarticulation of the humerus. *J. Morph.*, 62: 219-241.
- . 1949 Beryllium inhibition of regeneration. I. Morphological effects of Beryllium on amputated forelimbs of larval *Amblystoma*. *J. Morph.*, 84: 459-494.

PLATE 1

EXPLANATION OF FIGURES

(All plates are unretouched photographs of sections stained with Heidenhain's iron hematoxylin, $\times 150$ or $\times 500$.)

3 Section of the piece of epidermis stripped from the tail of the donor tadpole to be used as the transplant. This epidermis is three cells thick and possesses a heavy basement membrane. $\times 500$.

4 Longitudinal section of the proximal area of an experimental limb the day after operation. The epidermal transplant encircles the stripped limb at the thigh and knee. Hg 25, one day post-amputation. $\times 150$.

5 Regenerating limb in stage 1, the stage of epithelial migration. The flattened, migrating epithelial cells can be seen reaching the amputation surface. Hg 22, three days post-amputation. $\times 150$.

6 Proximal area (knee) of a regenerating experimental limb. The transplanted epithelium has migrated distally, leaving its basement membrane behind on the knee. Tf 35, 4 days post-amputation. $\times 150$.

7 Proximal area of another regenerating experimental limb. The transplant was curled back under itself. The epidermal cells which are thus placed internally in the limb disappear, as can be seen in this photograph. Hg 17, 5 days post-amputation. $\times 150$.

8 Higher power view of the distal area of the basement membrane shown in figure 7. (This section is several sections away from the one in fig. 7.) The epidermal cells caught inside the limb lose their typical characteristics and appear to dissociate inside the limb. Hg 17. $\times 500$.

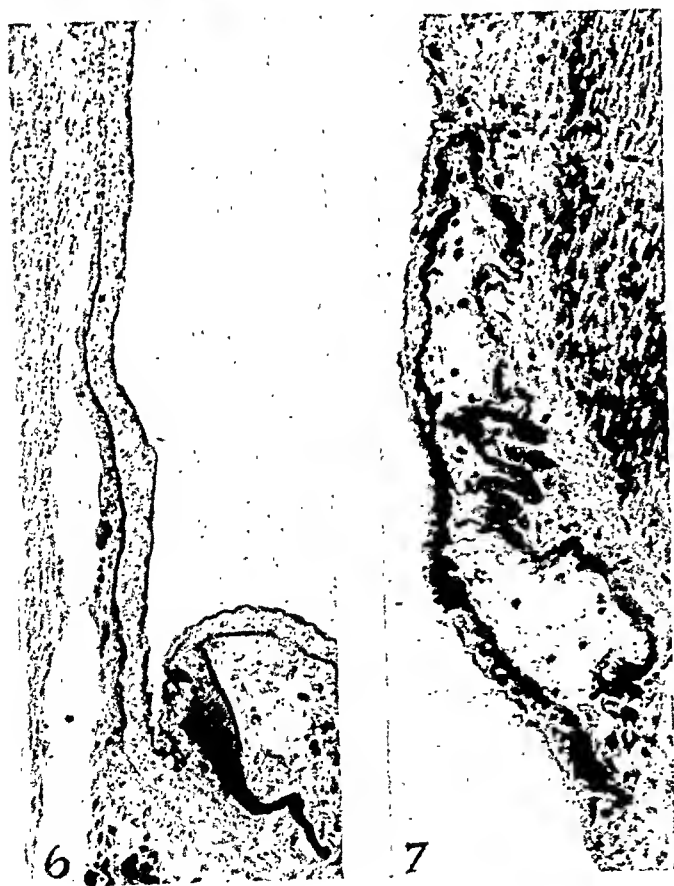


PLATE 2

EXPLANATION OF FIGURES

9 Regenerating limb in stage 2, the epidermal mound stage. The center of this thick pile of epidermis is beginning to disappear. The blastema contains only a few cells. Tf 43, 5 days post-amputation. $\times 150$.

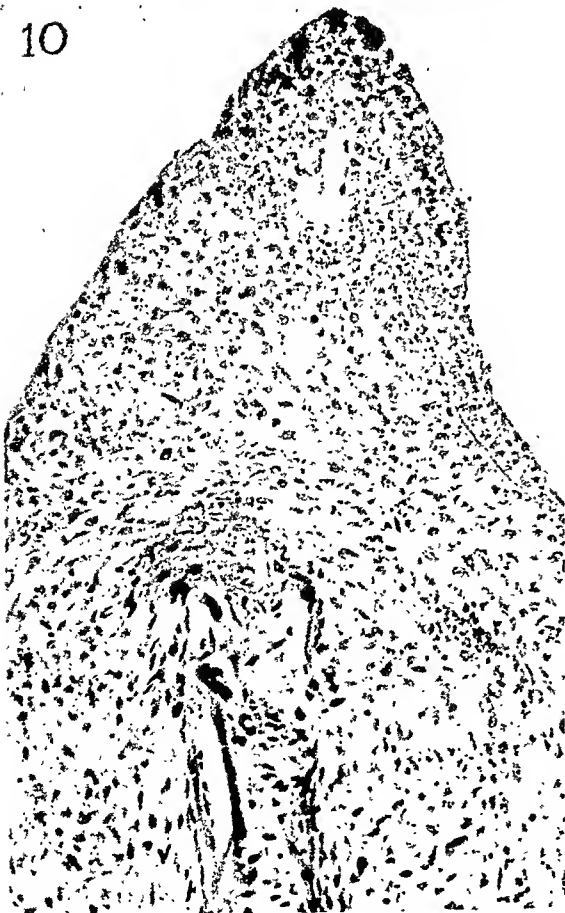
10 Regenerating limb in stage 3, the early blastema stage. The epidermal mound at the tip is reduced in thickness and contains darkly staining bodies of unknown nature. The blastema is loose in structure, and the blastema cells are large and variable in size. Little proliferation has occurred. Hg 18, 7 days post-amputation. $\times 150$.

11 Regenerating limb in stage 4, the stage of proliferation. The tip epidermis is several cells thick, possesses a thin basement membrane, and stains like the rest of the epithelium. The proliferating blastema has a compact structure, and the cells are smaller in size. Tf 2, 9 days post-amputation. $\times 150$.

9



10



11



PLATE 3

EXPLANATION OF FIGURES

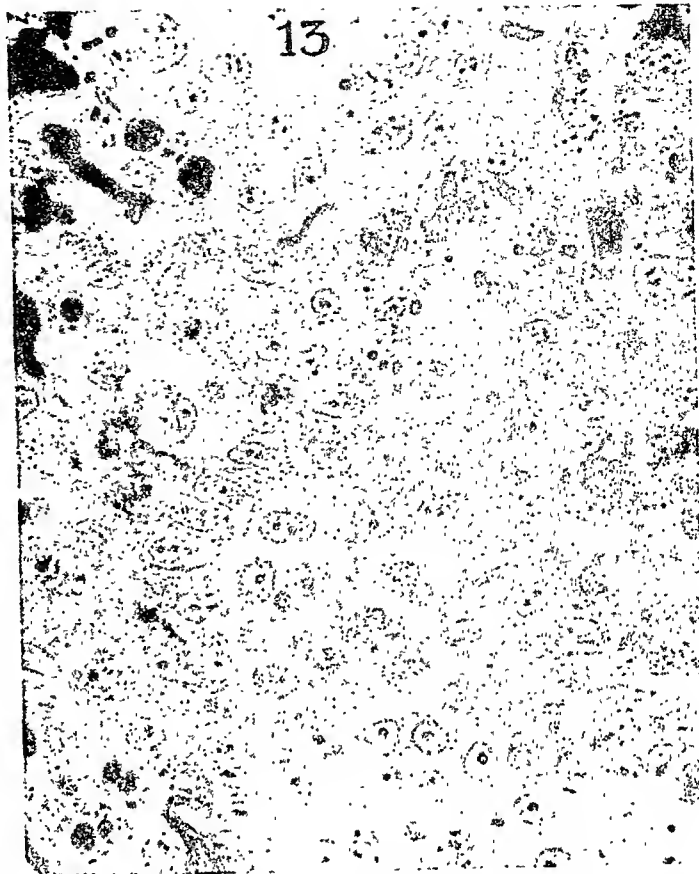
12 Another regenerating limb in stage 2. Tf 29, 7 days post-amputation. $\times 150$.

13 Higher power view of the boundary between tip epidermis and blastema in figure 12. The epidermal cells are becoming dissociated and appear to be entering the blastema. There is no demonstrable basement membrane under the tip epidermis in this stage. Tf 29. $\times 500$.

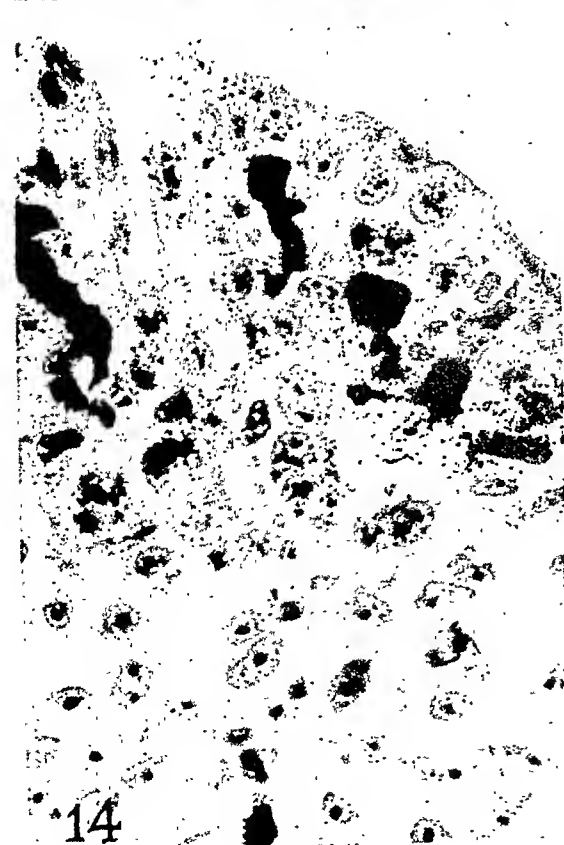
14 High power view of the tip of a regenerating limb late in stage 2. In this blastema, the majority of nuclei possess a large coalesced nucleolus, so that the maximum number of nucleoli cannot be determined. In such a case nucleoli cannot be used for identification of chromosome number. Hg 26, 5 days post-amputation. $\times 500$.

15 High power view of the tip of a regenerating triploid limb in stage 4. The nuclei possess one to three nucleoli. A number of triploid anaphases can be seen. Tf 1, control limb, 8 days post-amputation. $\times 500$.

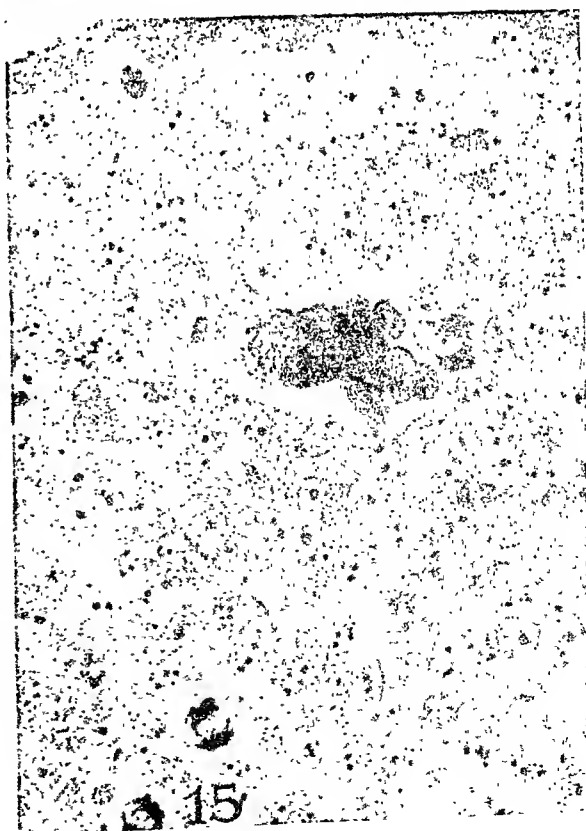
12



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14



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PLATE 4

EXPLANATION OF FIGURES

16 Proximal area of a regenerating triploid limb which received a haploid epidermal transplant. The basement membrane of the transplant can be seen to end in the lower middle of the photograph. The haploid epithelium which has migrated beyond its basement membrane is being invaded by phagocytes of the host. Tf 38, 5 days post-amputation. $\times 150$.

17 Higher power view of the same section shown in figure 16 taken from the area in which the basement membrane of the transplant terminates. The haploid nuclei are much smaller than the host nuclei (fig. 18) and possess one nucleolus. Half of two haploid anaphase figures can be seen in the epithelium in the middle of the photograph. The darkly staining nuclei under the basement membrane belong to phagocytes from the host. Tf 38. $\times 500$.

18 High power view of the host epidermis (triploid) in another section of the limb shown in figure 16. Compare the size of the triploid anaphases with the haploid in figure 17 and the diploid in figure 19. Tf 38. $\times 500$.

19 High power view of the control limb (diploid) of Hg 21. This regenerating limb is in stage 4, the stage of proliferation. The maximum number of nucleoli is two. Compare with figure 15, a regenerating triploid limb in stage 4. Hg 21, left limb, 6 days post-amputation. $\times 500$.

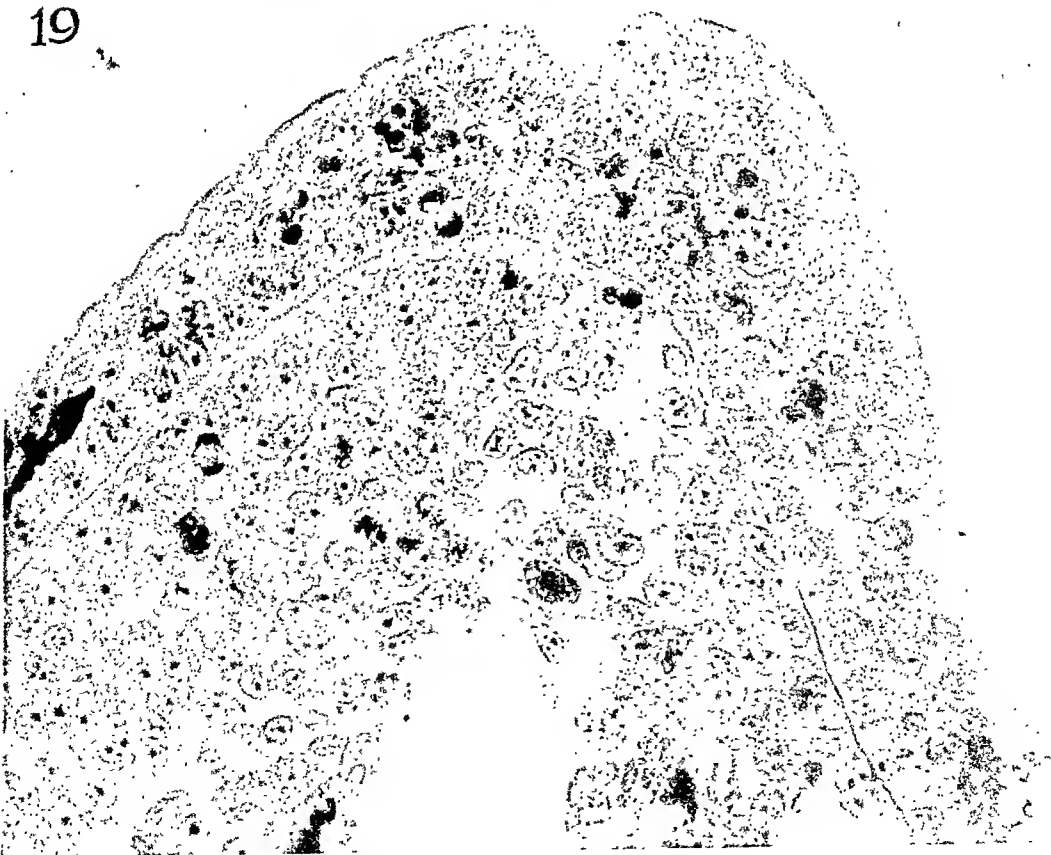
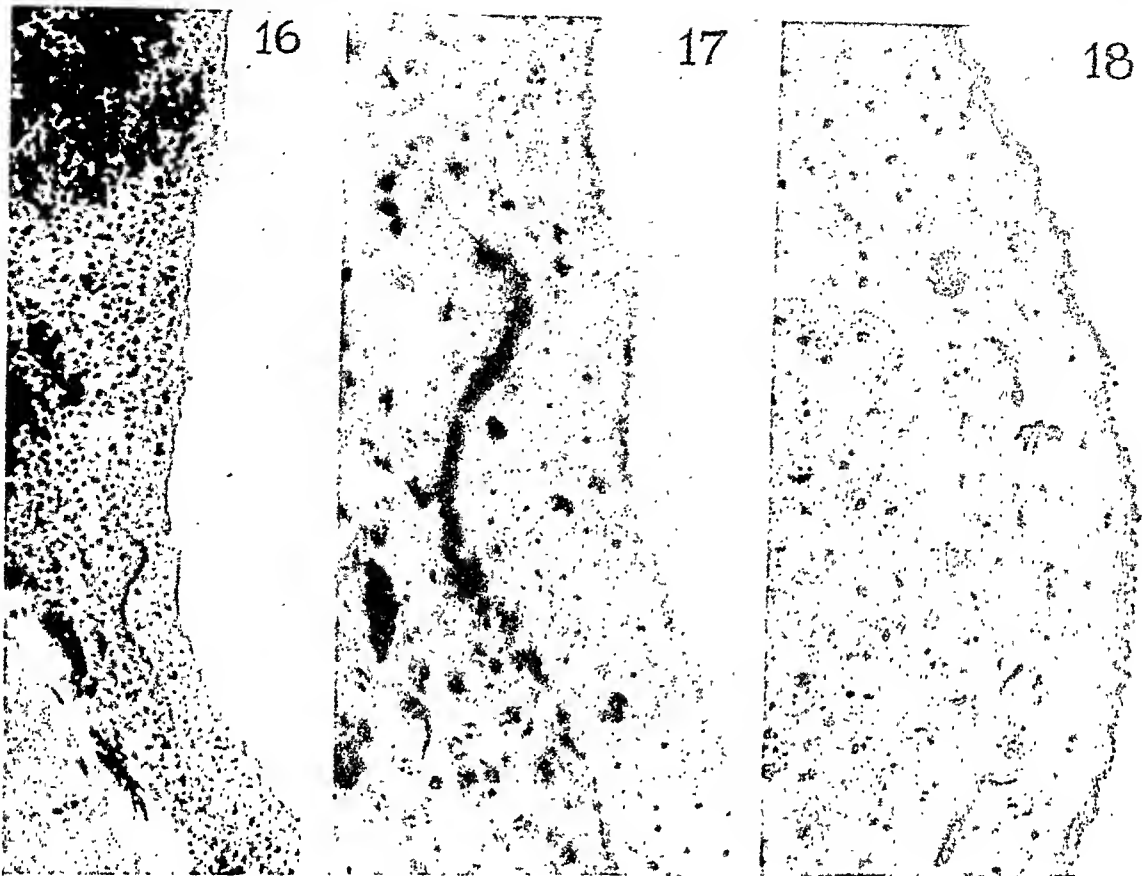


PLATE 5

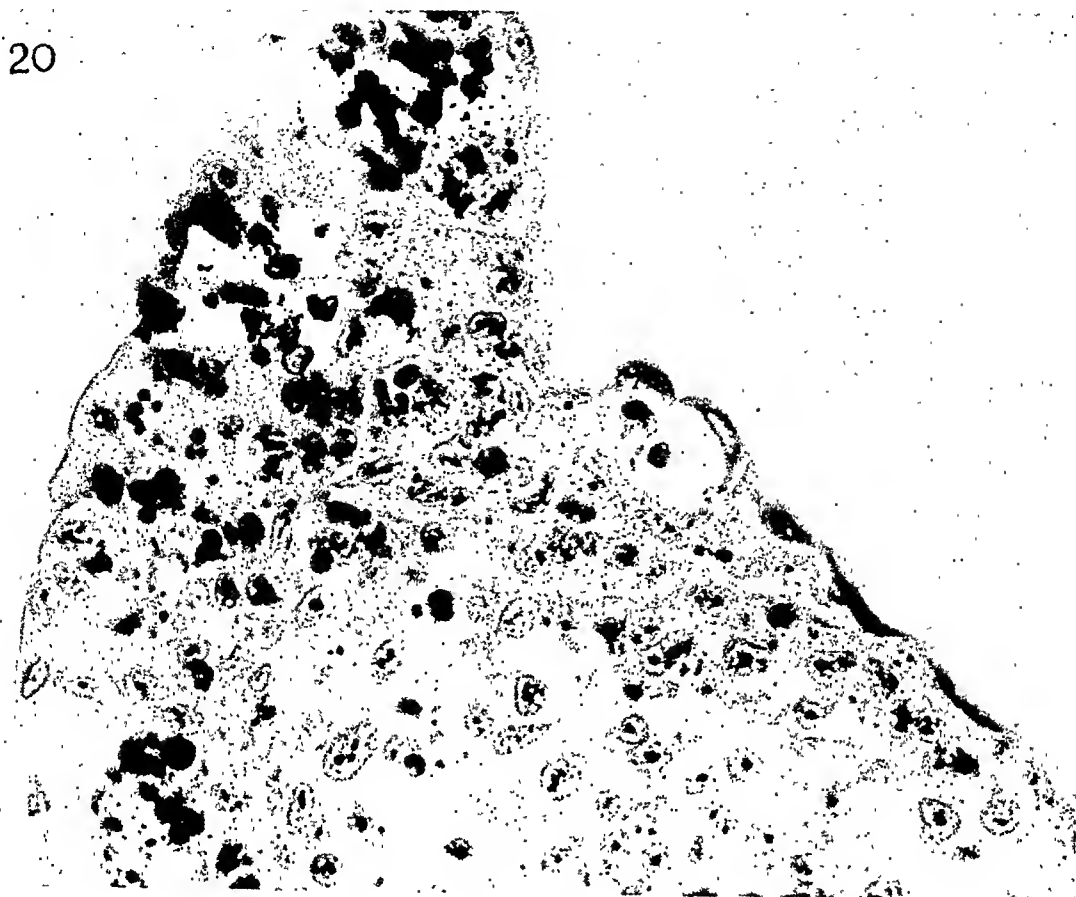
EXPLANATION OF FIGURES

20 High power view of the experimental limb of Hg 21. This limb received a triploid-tetraploid epidermal transplant. The large nucleus of a triploid cell with three nucleoli, which has come from the transplant, can be seen inside the blastema. This regenerating limb is in stage 3, the early blastema stage. Hg 21, right limb, 6 days post-amputation. $\times 500$.

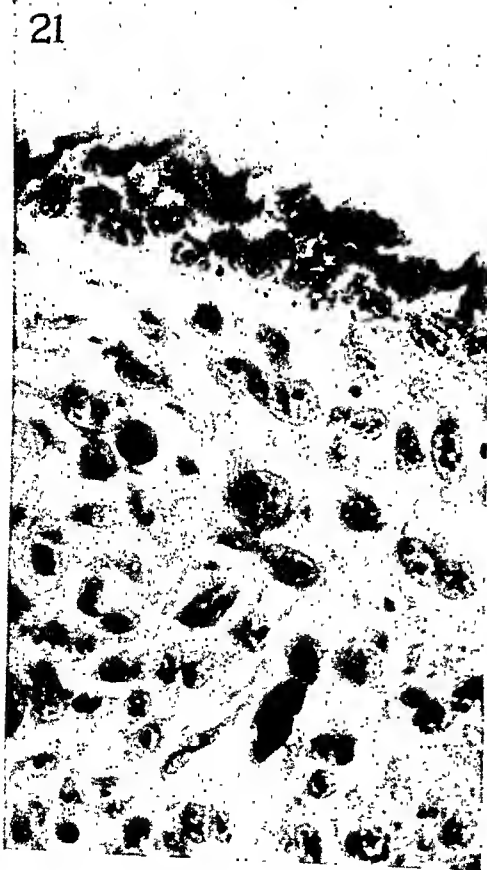
21 A different section of the experimental limb of Hg 21. This section is 23 sections away from the one in figure 20, through the blastema on one side of the epidermal mound. The large triploid cell in the upper center of the blastema has the diffuse cytoplasm and clear nucleus typical of the mesenchyme-like blastema cell, yet it has apparently originated from the epidermal transplant which this limb received. Hg 21, right limb. $\times 500$.

22 Another representative section of the experimental limb of Hg 21. This section is 18 sections from the one in figure 20 in the other direction from figure 21. A large tetraploid blastema cell with 4 regularly placed nucleoli can be seen. Such polyploid cells, which came from the transplant, are present throughout the blastema in almost every section. Hg 21, right limb. $\times 500$.

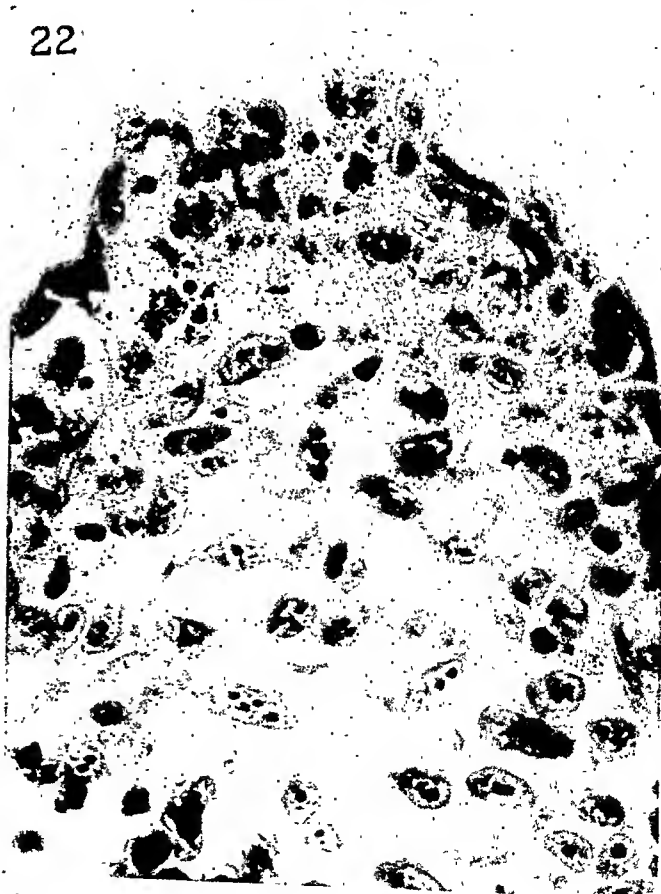
20



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HISTOPHYSIOLOGIC STUDIES ON SWEATING¹

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TEN FIGURES

INTRODUCTION

There are many ways to elicit sweating, and although the literature is replete with observations regarding it, few studies in the past have made any attempt to correlate the physiological phenomenon of sweating with the morphological changes which then occur in the structures involved (Bunting, Wislocki and Dempsey, '48; Olivet and Nauck, '30; Ring and Randall, '47). Sweating may be elicited by heat, by psychogenic stimuli, by the action of specific drugs, and by electrical stimulation of the nerve fibers supplying the glands. In addition, clinical observations also indicate that abnormal overstimulation of certain sympathetic but cholinergic nerves may induce profuse perspiration. The reaction of the gland during sweating appears to be the same regardless of the nature of the stimulus which produces the reaction. This study proposes to correlate sweating induced by heat and by drugs with histological and histophysiological changes in the glands.

The tissues for histological study were obtained from cats' paws in which sweating was induced either by drugs or by heat, and this was compared with similar material obtained from unstimulated, non-sweating paws and with ma-

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terial obtained after sweating had been stopped by the use of antidiaphoretic drugs. The histological material was studied in both unfixed frozen sections and in fixed serial sections.

METHODS

Sweating was produced in cats and, in a few instances, in rabbits by three different methods:

1. Animals anesthetized by various agents, and others which were unanesthetized were placed in a chamber in which temperature and relative humidity could be regulated within narrow limits. This chamber measured 7 feet high and 6 feet wide by 9 feet long. It was well insulated and well ventilated, and temperature and relative humidity were controlled from without the chamber. Temperatures could be controlled to $\pm 1^{\circ}\text{C}$., and relative humidity to within 5%. In general, the temperature was maintained at $48.9^{\circ}\text{C} \pm 1^{\circ}\text{C}$. with a relative humidity ranging from 9-14%. Rectal temperatures were periodically recorded in all of this group of animals with a "chemical" thermometer.

2. In the second method the carotid arteries were exposed, and the blood heated with an infra-red lamp placed 25 cm above the arteries, which with the underlying tissues were protected with moist cotton pads. There was no observable injury to either the underlying tissues or the vessels as a result of irradiation. Rectal temperatures were continuously recorded with a thermometer, accurate to 0.1°C .

3. Lastly, anesthetized animals were subjected to the intravenous injection of various doses of pilocarpine hydrochloride, an alkaloid possessing the property of stimulating all those tissues which are innervated by the parasympathetic nervous system, and of stimulating the sweat glands which, although apparently innervated by sympathetic nerve fibers, act as though they were innervated by parasympathetic fibers. Body temperatures were not recorded in these animals.

The animals which were placed in the heat chamber were anesthetized either with 30 mg/kg of pentobarbital sodium

administered intraperitoneally, or with $2.0 \text{ cm}^3/\text{kg}$ of paraldehyde or with paraldehyde and alcohol given orally on an empty stomach. Those animals in which sweating was induced by heating the carotid blood or by intravenous injection of pilocarpine hydrochloride were anesthetized with ether or with alcohol and ether. A few of the animals whose carotid blood was heated were anesthetized with pentobarbital sodium, but the results were unsatisfactory, for under this anesthetic, little or no sweating could be induced by drugs, by heating the carotid blood, or by heating the animals in the chamber.

When profuse sweating was observed, the paws were removed (the animals being still under anesthesia) and placed immediately either in physiological saline warmed to body temperature or in Helly's fixative. In the first instance, tissues were sectioned almost immediately with a freezing microtome at either 40 or 100μ in order to observe the ducts, or at 20μ in order to study their reactions to neutral methylene blue. Those tissues which were fixed in Helly's fluid were sectioned serially at 6 to 18μ and stained with Mallory's phosphotungstic acid hematoxylin. The same methods were used to study unstimulated, non-sweating glands and ducts. In 5 – 15 minutes following antidiaphoretic measures, when sweating had stopped, the remaining tissues were prepared by these same methods.

Three antidiaphoretic methods were employed in an attempt to terminate perspiration when it was most profuse: (1) Short arterial injection of a 5% aqueous solution of ferric chloride (0.1 cm^3 total dose). Short arterial injection is an injection into that artery which leads directly into the tissue being investigated and thus exposes the tissue to the injected material before that material is diffused throughout the organism.) (2) Intramuscular injections of various doses of atropine, an alkaloid which inhibits all cholinergic responses. (3). Intramuscular injection of alkalized agaric acid solutions. Agaric acid, $\begin{array}{c} \text{CH}_3 - \text{C}(\text{OH}) - \text{CH} \cdot \text{C}_{10}\text{H}_{23} \\ | \quad | \quad | \\ \text{COOH} \text{ COOH} \text{ COOH} \end{array}$, a little-used anhidrotic derived from the fungus *Polyporus officinalis*,

prevents, in man as well as in cats, the sweating which follows heat stimulation or the administration of cholinergic drugs. The peculiar feature of this drug is that, unlike atropine, it does not block other cholinergic responses (Sollmann, '48).

While there are several methods to estimate qualitatively and quantitatively the amount of sweat secreted, there are no previously reported color reactions to distinguish between a secreting and a non-secreting sweat gland. Sperling ('46) has shown that neutral solutions of methylene blue which almost instantaneously change into a leuco form in the presence of very small amounts of a strong reducing agent, such as sodium hydrosulfite, return as rapidly to the colored form when molecular oxygen is introduced. This instantaneous reversal can be continued as long as the reducing agent is present in the solution and may be utilized to study the activity of the sweat glands, by observing the color changes associated with the activity of the glands and ducts. Frozen sections, cut at 20 μ , of sweating and non-sweating paws were placed in solutions of the reduced, or leuco form of methylene blue and examined under the microscope. Similar sections from unstimulated, non-sweating paws were placed in such leuco solutions for about three minutes and then transferred to methylene blue solutions to which pilocarpine had been added (Sperling and King, '47).

In a number of frogs with profuse mucous skin secretions, successful attempts were made to stop these secretions by the intramuscular injection of atropine or of alkalinized agaric acid solution, or by the application of aluminum chloride to the surface of the skin. Histological studies of fixed skin sections were made during the secreting stage and following the administration of the above-named inhibiting agents.

PHYSIOLOGICAL OBSERVATIONS

Cats. Both carotids of 10 cats were exposed and irradiated as described in the previous section. Animals were anesthetized either by an intraperitoneal injection of 2.5 cm³/kg of a 20% solution of alcohol supplemented by ether, or with

ether alone. When surgical anesthesia was reached with muscular relaxation, a central pad was removed for study as outlined, rectal temperatures were recorded and irradiation started. The initial rectal temperatures in 10 cats so treated ranged between 37°C. and 37.5°C. Ten minutes after the start of irradiation, rectal body temperatures had risen to 38.5°C. , and continued to rise steadily during the next 10 minutes until a temperature of 40.5°C. was reached. At this time, pressure on the paw elicited slight moisture. Prior to the appearance of any sweat, the paws were quite warm to the touch. However, just before the body temperature reached 41°C. , profuse sweating occurred. At about 39°C. , hyperpnea

TABLE 1

The critical temperatures at which sweating occurred in cats following heating of the carotid arteries

| CAT NO. | INITIAL BODY TEMPERATURE $^{\circ}\text{C.}$ | SWEATING BODY TEMPERATURE $^{\circ}\text{C.}$ | MAXIMUM BODY TEMPERATURE $^{\circ}\text{C.}$ |
|---------|--|---|--|
| 8 | 37.8 | 40.8 | ... |
| 9 | 37.6 | 40.8 | 42.2 |
| 10 | 37.2 | ... | 40.0 |
| 11 | 37.8 | 40.6 | 41.7 |
| 12 | 38.0 | 40.8 | ... |

began and respiratory movements soon became too fast to be counted. When this occurred, the progressive rise in body temperature slowed. Depression of the hyperpnea by the administration of more ether was followed by the resumption of the relatively rapid rise in body temperature. Profuse salivation was almost constantly present. When sweating became profuse, a central pad of one of the paws was removed for study (table 1).

Large doses of phenolsulfonphthalein, 5 mg/kg, were intravenously injected into 5 cats, two of which were doubly nephrectomized. Carotid irradiation was then started and attempts were made to detect the dye in the profusely sweating paws of all the animals so treated. In no case could the

dye be recovered from the sweat secretion, while it could frequently be recovered from the saliva. Sweating was then inhibited by two means: 0.1 ml/kg of atropine was injected intramuscularly, and when all the paws became dry, which occurred in approximately 5 minutes, a central pad was removed. The other method consisted of a short arterial injection of 0.1 cm³ of a 5% solution of ferric chloride. The treated paw became dry almost immediately, while the others stopped sweating about 10 minutes later.

Fourteen cats were tested in the heat chamber. Four were anesthetized with paraldehyde (2 cm³/kg orally), one of which was premedicated with an intraperitoneal injection of alcohol in the same dose as described above. Two cats were anesthetized with 40 mg/kg of sodium pentobarbital given intraperitoneally. Two cats remained unanesthetized. Initial respiration rates and rectal temperatures were recorded in both the anesthetized and unanesthetized cats prior to being placed in the heat chamber. Initial rectal temperatures ranged between 38°C. and 39°C., and respiration between 19 and 50. The heat chamber was maintained at 49°C. and the relative humidity ranged between 10 and 14%. Within 15 minutes after being placed in the chamber, those cats which were anesthetized with paraldehyde or with alcohol and paraldehyde showed rectal temperatures of 40°C. and profuse sweating. Maximum rectal temperatures after exposure for one hour reached 43°C. One cat, which exhibited only slight sweating, died shortly after removal from the cabinet.

During the stay in the cabinet, hyperpnea, with respirations ranging from 100 to more than 200 per minute, was present along with profuse salivation. The two cats which were anesthetized with sodium pentobarbital showed no signs of sweating and only a slight degree of hyperpnea. In these, respiration was less than 70 per minute, and rectal temperatures rose to 42°C. in an hour. Shortly after these cats were removed from the cabinet, they showed signs of recovery from the anesthetic, and with the appearance of the recovery signs, profuse sweating began, and at that time body tem-

peratures began to fall slowly. Both animals survived. The two unanesthetized and otherwise untreated cats showed extreme hyperpnea within 30 minutes. Respiration was 200 per minute and soon exceeded this rate. Body temperature did not rise above 42°C., and at 40°C. profuse sweating and extreme salivation occurred. The animals licked themselves vigorously and soon had themselves soaked with moisture, mostly from the saliva. They were restless, prowled about the cabinet and made attempts to escape by jumping at the observation window. When released from the cabinet, body temperatures began to fall promptly and, apparently, no ill effects resulted in these animals.

Two cats were anesthetized with paraldehyde, and one with alcohol and paraldehyde. The three were then given 0.1 mg/kg of atropine intramuscularly. During the stay in the cabinet these cats exhibited only a moderate hyperpnea with respiration ranging between 60 and 80 per minute. There was no sweating and no salivation. In two of the cats, rectal temperatures reached 43°C. within 30 minutes and remained at that level. In the third, the temperature rose to 44°C. At the end of the hour all three of the animals died shortly after removal from the cabinet.

Three paraldehyde-anesthetized cats were given an intravenous injection of 100 mg/kg of agaric acid dissolved in a sodium hydroxide solution. The body temperatures of these cats rose in the same manner as those to which atropine had been administered. These cats also exhibited a lack of sweating. Although salivation appeared to be somewhat inhibited, it did not cease completely as it did with atropine, and a good degree of hyperpnea was present. Respiration in these cats reached 114 per minute. Although two of these three cats acquired a temperature of only 40°C. and a third of 43°C., all died shortly after removal from the cabinet (table 2).

Rabbits. Similar physiological observations were made on 6 unanesthetized rabbits which were placed in the heat cabinet. Three had previously been given a total dose of 0.5 mg of atropine. The untreated rabbits showed profuse salivation

and lip sweating, and survived even though the body temperatures went as high as $44^{\circ}\text{C}.$; this temperature was reached in about 50 minutes. Rectal temperatures in the atropinized rabbits rose from $41^{\circ}\text{C}.$ to $44^{\circ}\text{C}.$ Though exposed to heat

TABLE 2
Effects of external heat on body temperature

| CAT NO. | ANESTHETIC | ADDITIONAL DRUGS | INITIAL BODY TEMP. $^{\circ}\text{C}.$ | INITIAL RESPIRATION PER MIN. | SWEATING BODY TEMP. $^{\circ}\text{C}.$ | MAXIMUM BODY TEMP. $^{\circ}\text{C}.$ | MIN. SWEATING RESPIRATION PER MIN. |
|---------|-------------------------|------------------|--|------------------------------|---|--|------------------------------------|
| 33 | Alcohol and paraldehyde | None | 38 | 40 | 40.5 | 42.5 | 170 |
| 36 | Paraldehyde | None | 38 | 42 (irregular) | 41.5 | 41.5 | — ¹ |
| 39 | Paraldehyde | None | 39 | 16 | 42.0 | 43.0 | 185 |
| 41 | Paraldehyde | None | 39.5 | 45 | 43.2 | 43.2 (died) very slight sweating | 200 |
| 34 | Paraldehyde | Agaric acid | 38.2 | 42 | no sweating | 40 (died) | 90 |
| 38 | Paraldehyde | Agaric acid | 38 | 36 | no sweating | 43 (died) | 114 |
| 42 | Paraldehyde | Agaric acid | 38.2 | no record | no sweating | 40 (died) | no record |
| 35 | Paraldehyde and alcohol | Atropine | 37.3 | 52 | no sweating | 44 | 64 |
| 37 | Paraldehyde | Atropine | 37 | 50 | no sweating | 43 (died) | 84 |
| 40 | Paraldehyde | Atropine | 37 | 29 | no sweating | 43 (died) | 88 |
| 43 | Pentobarbital sodium | None | 39 | 19 | no sweating | 42.5 | 68 |
| 44 | Pentobarbital sodium | None | 39 | 13 | no sweating | 42.5 | 63 |
| 45 | None | None | 39 | 28 | 40.0 | 41.5 | — ¹ |
| 46 | None | None | 39 | 33 | 40.5 | 42 | — ¹ |

¹ Excessive rate — not counted.

for about 30 minutes there was no salivation and no lip sweating. Two of these rabbits died, one in convulsions. The third survived, but had a violent convulsion. In all cases, respiration was too rapid to count during the stay in the

sweat cabinet. In rabbits, the respiratory rates are normally high, and prior to being placed in the sweat cabinet, these ranged between 100 and 150 per minute.

It can be seen from these experiments on cats and rabbits that in mammals, hyperpnea, the production of sweat, and profuse salivation constitute a definite mechanism for the loss of body heat. This loss is reflected in a less rapid rise of body temperature which does not become as high as in those animals in which sweating is inhibited. The inhibition of these mechanisms may well lead to a fatal outcome. Animals narcotized with sodium pentobarbital, where both sweating and hyperpnea were inhibited, did not exhibit a rise in body temperature as high as observed in those which were anesthetized with other anesthetics. This may be attributed to a depression of the central heat-production mechanisms. It is to be noted that those cats which were anesthetized appeared to show a deepening of the anesthesia during their stay in the cabinet, although there was no further administration of any anesthetic agent.

Frogs. To affect the respiratory function of the skin, three frogs were placed in a 10% aqueous solution of aluminum chloride. Three others were injected intramuscularly with a total dose of 0.25 mg of atropine, and a third group of three was injected with a total dose of 25 mg of agaric acid. Shortly after each of these treatments, the skins became dry, due to lack of mucous secretion, and this drying was shortly followed by death. As will be shown below, the only observable change appeared to be the inhibition of secretion by the mucous glands.

HISTOPHYSIOLOGY

The central pad of a paw from each of 4 unstimulated, non-sweating, ether-anesthetized cats was removed, immediately placed in saline warmed to body temperature, and within a minute or two after removal frozen sections were made with a freezing microtome at 20 μ . The sections were then placed in neutral leuco methylene blue solutions warmed to body temperature for three minutes and examined under the micro-

scope. In each of the 4 cats, the glomeruli and proximal portions of the sweat glands stained a deep blue. The same cats were then injected with a total of 2.5 mg of pilocarpine. When profuse sweating occurred, the central pad of another paw was removed and immediately placed in warm saline. Sections made on the freezing microtome were placed in a neutral leuco methylene blue solution, and studied with the microscope. In these sections, the glomeruli and the proximal portions of the ducts remained either completely unstained or at best took on a very faint stain. Similar sections in one cat which was atropinized, and in which sweating had stopped, stained with definitely less intensity than that shown by the unstimulated, non-sweating paw.

Sections of central pads from the paws of three additional unstimulated, non-sweating anesthetized cats, prepared and stained as described, were placed in a saline methylene blue solution to which sufficient pilocarpine had been added to make a 1% solution. Of sections from a total of 7 pads from two cats, 6 showed definite partial destaining; none showed complete decolorization, and in one, destaining in some of the sections was questionable (cats 30 and 31; table 3).

In another cat (cat 32) in which non-sweating central pads were removed and sectioned, destaining could not be obtained at all in the pilocarpine solutions. This cat was then injected with a total of 18 mg of pilocarpine, a dose more than 7 times that usually required to elicit profuse sweat. There were no signs of sweating, although all other signs of cholinergic responses were present, such as profuse salivation and lachrimation, and involuntary defecation and micturition. Frozen sections of the paw after this huge dose still stained a deep blue after immersion in methylene blue. Obviously, the sweat glands of this cat were non-functional. Occasionally, cats have been observed from which sweating cannot be elicited. The literature refers to this phenomenon and states that the lack of sweat is due to hyperkeratinization of the paw (Coon and Rothman, '41). From the above results, it can be seen that this is probably not the case, but

TABLE 3

Reactions of frozen sections of tissues taken from sweating and non-sweating cats to leuco methylene blue solutions

| CAT NO. | TREATMENT | PAW | NEUTRAL LEUCO | METHYLENE BLUE LEUCO PILO. |
|---------|---------------------|-----|------------------|-------------------------------|
| 26 | None | 1 | ++++ | |
| | Pilocarpine, 2.5 mg | 2 | — + | |
| 27 | None | 1 | ++ | |
| | Pilocarpine, 2.5 mg | 2 | — | |
| 28 | None | 1 | ++++ | |
| | Pilocarpine, 2.5 mg | 2 | — | |
| 29 | None | 1 | ++++ | |
| | Pilocarpine, 2.5 mg | 2 | — | |
| | Atropine | 3 | ++ | |
| 30 | None | 1 | ++++ ----- | ++ |
| | | | ++++ ----- | +++? |
| | | 2 | | ± |
| 31 | None | 1 | ++++ ----- | ++ |
| | | 1 | ± | |
| | | 2 | ++++ | + ± |
| | | 2 | | — |
| 32 | None | 1 | ++++ ----- | ++++ |
| | | 1 | | ++++ |
| | | 1 | | |
| | | 2 | ++++ | |
| | | 2 | | ++++ |
| | | 2 | | |
| | | 3 | ++++ | |
| | | 3 | | ++++ |
| | | 3 | | |
| | Pilocarpine, 18 mg | 4 | ++++ | ++++ |
| | | 4 | ++++ | ++++ |
| | | 4 | ++++ | ++++ |

+ = degree of duration of maintaining the dye in its leuco form.

— = conversion of the leuco form to the blue color.

the absence of sweating, regardless of the stimulation, is as yet unexplained.

Serial sections were prepared from paws which exhibited profuse sweating as the result of administration of pilocarpine or as a result of heating the carotid blood. Similar sections were made of unstimulated, non-sweating paws and of paws in which sweating had been stopped by the administration of atropine or of ferrie ehloride. The sections were fixed in Helly's fluid, imbedded in paraffin, serially sectioned at 6 to 18 μ and stained with Mallory's phosphotungstic acid hematoxylin.

The histology of the sweat gland has been described in a number of papers. All seemed agreed with regard to the ecerine type of sweat gland, which is the kind found in cats' paws. These consist of a secretory portion, the glomerulus, in which there is a pseudo-stratified epithelium, and a duct in which the epithelium changes from columnar to cuboidal as it approaches the stratum germinativum and, finally, becomes quite flattened. The whole is invested in a fibrous envelope. The lumen of the sweat gland sends out numerous intercellular canals which are mostly confined to the glomerulus. Myoepithelial elements (hereafter abbreviated, "elements") have been described as elongated fibrillar cells, following, more or less, the long axis of the tubule of the glomerulus and extending into the proximal portion of the duct. They have been described as being fibrillar, 40-100 μ long and 6-10 μ wide. Heynold (1874) describes the elements as smooth muscle cells and credits Kölliker with first describing them in 1869. (Hoepke, '27 credits Kölliker with naming these myoepithelial elements in 1889.) Sheldon ('41) describes their embryological origin but quotes no source for this description. All of these authors agree that the elements are elongated cells which vary within the limits stated above and that each consists of an elongated central body with a dense nucleus. They agree that the elements lie between the glandular epithelium and the basement membrane along the intercellular canals, and follow, more or less, the long axis of the tubule

of the glomerulus and extend into the proximal portion of the duct.

Actually, the length of these cells is due mainly to numerous long processes, somewhat resembling pseudopodia, which extend from each end of the cell body (see figs. 1 and 2). All of the processes do not originate at the same level and often bend abruptly into other planes. Since the elements lie between the glandular epithelium and the basement membrane along the intercellular canals, and since the glandular epithelium actually straddles the extended process, retraction of these serves to increase the length of the intercellular canals. The function which has been ascribed to the elements is that of contraction in order to express the sweat out of the gland (Renaut, 1894). During sweating, whether heat-induced or drug-induced, the elements undergo definite diminution in size. This shrinkage does not appear to be contractile in nature, despite the fact that the elements are fibrillar and the fibrils resemble those of smooth muscle (Bunting, Wislocki and Dempsey, '48).

Outline tracings were made of serial sections, magnified by 450 diameters, of entire glomeruli and associated myoepithelial elements of unstimulated, non-sweating glands, of glands in which sweating was induced by pilocarpine, of glands in which sweating was induced by heating the carotids, and of glands in which sweating had been stopped by the administration of atropine. Morphological observations were also made of glands in which sweating had been stopped by the short arterial injection of ferric chloride, but tracings were not made of these.

The outline tracings of the surface areas of these glands in sections were measured with a planimeter and from this area was deducted the area of the lumen. The areas thus measured for each gland were summated. Similar measurements were made of the myoepithelial elements. The ratio, in terms of per cent, between the area of the elements and the surface area of the gland in section was then calculated. In the unstimulated, non-sweating gland, the elements oc-

occupy approximately 20% of the sectional area of the gland. In those glands in which sweating was produced by the administration of pilocarpine, the elements occupy slightly more than 3% of the sectional area. In those glands in which sweating was produced by heating the carotid arteries, the elements occupy approximately 5% of the sectional area. Finally, in those glands in which sweating had occurred, and in which this had been stopped by atropine administration, the elements occupy about 6% of the sectional area. These measurements indicate that when sweating occurs, the myoepithelial elements lose about 80% of their volume. If we consider that it is most likely that this loss is due to the loss of water, especially in view of the fact that muscle tissue and probably all other tissue contains between 85% and 99% osmotically active water (Fenn, '36; Hunter and Parpart, '38), and further, that the elements lie along the intercellular canals, it becomes probable that at least one of the functions of the elements is to contribute to the initial flow of sweat, for they remain small during the period of glandular activity.

In unstimulated, non-sweating glands, the elements are approximately 100μ long and about 10μ wide. In the sweating gland, they are only about 50μ long and 6μ wide. From this, and from the above measurements, it can be seen that actually these elements lose a considerable portion of their volume. The degree of this loss is best seen in table 4. During the diminution in size of the elements, many of the processes which extend from each end of the central portion of the element become retracted, so that the element is left with one or two processes which in themselves have lost much of their bulk (see fig. 3). The total loss in volume of all of the elements per gland is of the order of magnitude of 0.05 mm^3 . This represents a considerable volume of fluid when we consider the large number of glands involved. It would represent a considerable contribution to the initial outflow of sweat, and may thus represent an emergency mechanism for the rapid outpouring of perspiration. None of these observations indicates active contraction of the elements.

TABLE 4

Ratios of myoepithelial elements to sweat gland areas in cats

| CAT NO. | GLAND NO. | GLAND AREA | ELEMENT AREA | PER CENT ELEMENT GLAND |
|--------------------------------|-----------|------------|--------------|---------------------------|
| <i>Non-sweating</i> | | | | |
| 1 | 1 | 100.61 | 18.76 | 17.7 |
| 1 | 2 | 45.11 | 8.37 | 18.7 |
| 1 | 3 | 40.93 | 8.50 | 19.2 |
| 1 | 4 | 60.60 | 11.92 | 19.7 |
| 1 | 5 | 43.58 | 9.25 | 21.2 |
| 11 | 6 | 50.48 | 11.80 | 23.5 |
| 11 | 7 | 47.66 | 10.05 | 21.1 |
| <i>Sweating (pilocarpine)</i> | | | | |
| 1 | 1 | 94.20 | 3.86 | 4.1 |
| 1 | 2 | 87.90 | 3.64 | 4.1 |
| 1 | 3 | 58.10 | 2.06 | 3.5 |
| 1 | 4 | 69.08 | 2.70 | 3.9 |
| 21 | 5 | 36.10 | 1.31 | 3.6 |
| 21 | 6 | 56.58 | 1.69 | 3.0 |
| 21 | 7 | 31.62 | 0.72 | 2.3 |
| 21 | 8 | 45.08 | 1.04 | 2.2 |
| <i>Non-sweating (atropine)</i> | | | | |
| 20 | 1 | 46.46 | 3.60 | 7.7 |
| 20 | 2 | 25.12 | 1.52 | 6.0 |
| 20 | 3 | 121.36 | 6.60 | 5.5 |
| 20 | 4 | 122.98 | 6.80 | 5.5 |
| 20 | 5 | 103.92 | 6.80 | 6.6 |
| 20 | 6 | 89.42 | 4.40 | 5.1 |
| 20 | 7 | 27.52 | 2.00 | 7.3 |
| 23 | 8 | 39.68 | 1.36 | 3.0 |
| 23 | 9 | 56.98 | 2.60 | 5.0 |
| 23 | 10 | 82.56 | 5.60 | 6.8 |
| <i>Heat sweating</i> | | | | |
| 25 | 1 | 82.29 | 5.14 | 6.2 |
| 25 | 2 | 81.96 | 3.47 | 4.2 |
| 25 | 3 | 48.33 | 2.10 | 4.4 |
| 25 | 4 | 70.76 | 2.74 | 3.9 |
| 25 | 5 | 113.92 | 4.48 | 3.9 |
| 25 | 6 | 139.53 | 7.45 | 5.3 |
| 12 | 7 | 42.82 | 3.2 | 7.5 |
| 12 | 8 | 86.75 | 7.26 | 8.4 |

Both pilocarpine and the irradiation of the carotid arteries appear to stimulate the elements to approximately the same extent. Atropinization at best can only be said to tend to initiate the return of the elements to the non-sweating state. It does not appear to produce the contraction seen by Olivet and Nauck ('30). Furthermore the diminution in size is quite evident after the administration of pilocarpine, although Olivet and Nauck observed no effect after the administration of this drug. It is, however, to be pointed out that these investigators reported no measurements, and they used human axillary glands which are mostly of the apocrine type in which the elements are closely connected with each other in contrast to the isolated elements found in the eccrine glands.

After the administration of ferric chloride, the elements appear smaller than ever (see fig. 4). This is to be expected for the hygroscopic nature of this reagent would tend to remove any remaining free fluid from both the elements and the glandular epithelium.

Frozen, unfixed sections, cut at 40-100 μ , and which were studied both in the unstained condition and when stained with methylene blue and eosin, indicate that the portion of the duct in the corneum lying between the stratum germinativum and the pore undergoes changes during the process of sweating. In the non-sweating paw, this portion of the duct is a rather tightly coiled spiral whose diameter remains fairly constant (see fig. 5) until it approaches the pore where it assumes the shape of a funnel with the base at the opening. The diameters range from 3.5 μ in some ducts to 10 μ in others, while the funnel may vary from 14 to 16 μ . During sweating, however, the duct loses the spiral form and becomes a loosely convoluted tube (see fig. 6). All evidences of the spiral disappear. Local enlargements resembling ampullae are present and can be seen in the figure. The unexpanded portions of the tube and the funnel have the same diameter as that found in the non-sweating duct, but the ampullae may have a diameter of two to 4 times as great as the other portions of the tube and may often be as wide as the funnel. It thus appears

that sweating is not a continuous flow from any individual duct, but rather that individual droplets follow each other at irregular intervals, and that the ampullae represent these droplets.

The reactions of the skin of the frog to substances which inhibit secretion are quite characteristic. In the untreated frog, the mucous glands are large, spherical, with a basement membrane upon which rests an epithelium. The nuclei of this epithelium are close to base of the cells which are greatly elongated, have no clear border, and from which long strands of mucus radiate towards the duct (see fig. 7). The gland is a simple structure and opens directly to the surface of the skin by means of a very short straight duct. When, however, the skin is treated with alum, the cells become much shorter so that the nuclei occupy most of the entire cell and the cell outlines become quite distinct. The long mucous threads disappear almost completely.

When the animals have been treated with agaric acid (see fig. 8) or with atropine (see fig. 9), the epithelial cells do not become as shortened as when they have been treated with aluminum chloride (fig. 10), and there is somewhat more mucous present, but definite cell outlines are evident. Their appearance is transitional between that seen in the untreated gland, and that seen after aluminum administration. From this it is evident that the inhibiting agents act upon the glandular epithelium rather than on the duct. Thus, aluminum does not act as an astringent to close the duct, but acts directly on the glandular epithelium. Similar conclusions can be drawn from the study of cat preparations which show that although the condition of the duct is dependent upon the production of sweat, sweat production is independent of the condition of the duct. Modification of glandular activity must result from effects exerted upon the gland itself.

DISCUSSION

Sweating, one of the most effective of heat dissipating mechanisms, accounts for the loss of a great deal of body

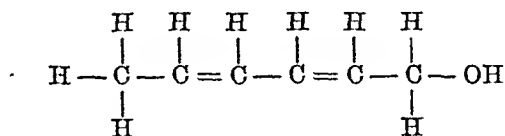
fluid. It should be pointed out, however, that as with many of the body functions, sweating may also occur when there is no particular need to maintain optimal body temperatures. It should also be pointed out that under circumstances where the loss of fluid is desirable, for reasons other than thermoregulation, sweating does not occur unless artificially stimulated. The emotional sweating is such conditions as fear; the gustatory sweating, particularly about the lips and nose, associated with the consumption of highly spiced foods; the cold, clammy sweat, often accompanied by nausea, vomiting, and fainting, in shock; and the frequently observed pathological sweating caused by obscure nervous lesions wherein local areas of a greater or lesser extent sweat profusely without apparent stimulation, are all illustrative of unnecessary sweating. On the other hand, when sweating would be highly desirable to eliminate metabolites, such as in cases of renal failure, sweating does not begin to operate automatically, but must be artificially stimulated by the use of diaphoretic drugs.

Relatively few species of mammals possess widespread functional sweat glands, which is a very peculiar phenomenon in view of the comparative anatomical evidence that all mammary glands are modified sweat glands (Hyman, '42). Among those species which do possess sweat glands, there are some in which these glands are limited to only a very small area of the body, e.g., the foot pads of the cat's paw, and the lips of the rabbit. Others, as man and the horse, may have sweat glands distributed over the whole body.

Among the important mechanisms by which heat loss may be accomplished in mammals are perspiration, salivation and hyperpnea. In the animals here investigated, in which sweat was produced by heating the carotids and which were under ether anesthesia, it required the depression of the extreme hyperpnea to elicit profuse sweating, although salivation was present constantly. Therefore, hyperpnea appears to be an efficient and potent method for promoting heat loss, for, as has been shown, progressive rise in body temperature is slowed when the hyperpnea becomes extreme. Sweating and saliva-

tion by themselves, are, however, sufficient to prevent a fatal outcome in some cases of overheating. It appears, therefore, that sweating, even in such a limited area as the cat's paw, is an important factor in preserving homoiothermy. Homoiothermy is, after all, only a relative term, since rabbits and cats show significant rises in body temperatures when placed in the heat chamber, or following irradiation of the exposed carotid arteries in the cat. Similar observations have been made in man and the dog. In the heat chamber, sweating occurs at somewhat lower temperatures than following heating of the carotid blood.

Heating the carotid blood elicits sweating at remarkably constant body temperatures, $40.5^{\circ}\text{C.} \pm 0.1^{\circ}\text{C.}$ This result suggests that sweating may be induced by the heating of thermoregulatory nervous centers. Similar conclusions were drawn by Gurney and Bunnell ('42), in human subjects in which sweating was induced in regions remote from anesthetized areas to which heat was applied. However, there is recent evidence that limited areas of sweat glands themselves respond both to locally applied heat in sympathectomized human subjects (Gurney and Bunnell, '42) and to the local application of hexadienol, which also produces a local sweat response (Koppanyi, '45). Hexadienol is a diene-alcohol with the tentative formula:



When the heat-regulating centers are depressed by certain anesthetics, such as the barbiturates, the animal becomes less sensitive to external heat, including irradiation of the common carotid artery. Barbiturates also interfere, in the cat at any rate, with sweating produced by cholinotropic drugs, indicating some action directly on the glands. Atropine and other belladonna alkaloids, as well as agaric acid, act by inhibition of the peripheral sweating mechanism and, indeed, interfere with the survival of animals in both the sweating

chamber and following irradiation of the common carotid arteries. Incidentally, it was observed that when body temperatures in cats and rabbits reached 41.1°C ., normal body temperatures could not be easily restored and removal of the heat source had no immediate effect upon the continued progressive rise of body temperature. When the rectal temperature reached levels higher than 43°C ., the animals failed to recover. The indications are that this progressive increase in body temperature, even when the animal is removed from the high temperature environment, is due to overstimulation of the central heat regulating mechanism, by either direct heat action or some other unknown factor. Careful autopsy in those animals which died revealed no detectable morphological changes either in the central nervous system or in the blood, nor was the coagulation of the blood affected.

Sweat seems to be more than an ultrafiltrate. Secretion pressures of 250 mm of mercury have been reported in a single duct of a gland (Best and Taylor, '45). The composition of sweat, nevertheless, suggests that it must be at least in part of the nature of an ultrafiltrate, for there are no constituents in sweat which do not occur in the blood (its pH, as analyzed in the duct, is slightly alkaline like the blood), and various substances, such as the bromides and iodides, can be recovered from sweat (Sollmann, '48). There is also the possibility that the proximal portion of the duct is active during sweating. This is shown by the methylene blue test for sweat gland activity. The lack of coloration of the leuco compound in the active glands and in the proximal portion of the duct of these glands is probably due to the utilization of oxygen by the actively functioning tissues. Ring and Randall ('47) present pictures which appear to show exhaustion of cellular elements as a result of sweating in the rat. The pictures which they use for illustration may well be of sections from the proximal portion of the duct, for these show a single epithelial layer, while the glomerulus, in cats at any rate, has a pseudostratified epithelium. Similar changes in the epithelium could not be recognized in the glomerulus in

our preparations. Furthermore, the morphological evidence, as shown by the measurements, indicates that the adnexa of the sweat gland (the so-called myoepithelial elements) partake, at least initially, in the production of sweat, along with the glomerulus and the proximal portion of the duct.

The change in size of the myoepithelial elements during the process of sweating can be accounted for by the loss of water from these fibrillar structures, for most tissues contain very large amounts of osmotically active water, and muscle tissue, as shown by Fenn ('36) and by Hunter ('36) and by Hunter and Parpart ('38), contains 85% to 99% water, an amount of which is probably of the same order of magnitude as that found in other tissues. The reduction in size of the myoepithelial elements is great and within the limits of the contained unbound water. The location of the elements is such that water would have no difficulty leaving them and entering the lumen of the sweat gland. Regardless of whether sweating is pilocarpine-induced, or whether it is induced by heating the carotids, the myoepithelial elements show similar changes. Olivet and Nauck ('30) could find no changes in the myoepithelial elements of human axillary glands after sweating induced by pilocarpine. They did find a reduction in size of the elements after the administration of atropine. Their findings were based upon serial sections, but measurements were not reported. In the experimental observations given above, there are indications that atropinized animals show myoepithelial elements which are beginning to return to the original non-sweating state. Ring and Randall ('47), using rats, discuss the appearance of the epithelium under conditions of sweating and non-sweating, but apparently made no observations of their own on the myoepithelial elements.

In the past (Renaut, 1894), it had been supposed that the function of the myoepithelial elements was to contract and thus aid in the discharge of sweat from the gland, and our own preliminary observations appeared to confirm this (Sperling and Koppanyi, '46), for the literature up to now has considered these cells as being smooth muscle (Heynold, 1874;

Hoepke, '27; Sheldon, '41; Maximow and Bloom, '48). That this is open to question is indicated by the measurements seen in table 4. It is probable that one of the functions, at least, of the myoepithelial element is to provide fluid for an immediate initial outflow of sweat, for calculation will show that their change in size from non-sweating to sweating represents a volume of the order of magnitude of 0.05 mm^3 . The nature of the fibrillar structures in the myoepithelial elements and their function remain to be elucidated. They appear to have some resemblance to the fibrils found in smooth muscle (Bunting, Wislocki and Dempsey, '48), but whether they actually contract is problematical. Studies done on striated muscle, which show that immersion in isotonic potassium solutions cause swelling of the fibers, and in isotonic sodium solutions, replacement of the intracellular potassium (Steinbach, '47), indicate changes which are the opposite of those which occur in the myoepithelial elements during sweating. Singh ('44) reports similar swelling with potassium and lesser swelling with sodium of invertebrate smooth muscle. Furthermore, the magnitude of change in volume of muscle during contraction is very small and is due to physicochemical changes which result from the metabolic activities associated with contraction (Meyerhof and Möhle, '33; Meyerhof, '47; Fischer and Morocz, '41). In striated muscle, Maximow and Bloom ('48) state that there is a loss of volume of 0.5%, and Fischer ('36) reports that measurements of contracting invertebrate smooth muscle show that there is a diminution of size ranging between $0.000,005^{\text{th}}$ and $0.000,002^{\text{th}}$ of the volume of the muscle.

The pressures of 250 mm of mercury which have been recorded from the lumina of sweat glands would be expected to produce some easily observable effect, and these pressures are probably responsible for the loss of the spiral form during sweating in the portion of the duct which lies in the stratum corneum. During sweating the lumen of this portion of the duct exhibits dilatations at irregularly spaced intervals. These probably represent individual droplets of sweat. The two implications here are that some pressure is necessary

to propel these droplets, and that the outpour of sweat is not a continuous flow, but rather a succession of droplets passing through the duct.

In a qualitative series of observations in which a short arterial injection of ferric chloride (fig. 4) was made into the sweating paw, the myoepithelial elements diminished in size, apparently to a degree even smaller than that found during sweating. Inasmuch as ferric chloride is a hygroscopic agent, it can be assumed that water was withdrawn from both the gland and the elements, and that as sweating ceased, the gland began to become dehydrated. The passage of water in this case would be away from the gland into the surrounding tissue fluid.

From the foregoing, it appears that the production of sweat involves at least two, and possibly three, sources, all reacting to the same stimuli at the same time. These three sources are: (1) the so-called myoepithelial elements; (2) the glomerulus of the sweat gland; and (3) the proximal portion of the duct of the sweat gland. These structures apparently have the same innervation since cholinotropic drugs induce changes in the glomerular function and in the appearance of myoepithelial elements simultaneously.

Also, it appears that the term "myoepithelial element" is something of a misnomer for, although these bodies may have an epithelial origin, their chief function does not appear to be myoid. Perhaps merely calling them fibrillar bodies would be more appropriate.

The inhibition of sweating can be accomplished, apparently, only by the inhibition of the glandular epithelium and its adnexa. Mere closure of the ducts is ineffective, as has been shown by Shelley, Horvath and Horvath ('48) who produced extensive vesiculation in man, after closing the ducts by iontophoretic means. Much the same situation appears to be true in the inhibition of mucous secretion in the frog skin. The glandular epithelium is inhibited by the three anhidrotics which were used. Thus, in the frog, an astringent, such as aluminum chloride, when applied to the body surface, can

penetrate and inactivate the epithelium, while in mammals, when applied locally, astringents ordinarily cannot reach the gland and are, therefore, unable to stop sweating. Where they can penetrate, as following the short arterial injection of iron chloride, they may inhibit sweating.

SUMMARY

1. In cats, heat-sweating occurs only when the body temperature reaches a critical level ($40-41^{\circ}\text{C}.$). This is accompanied by hyperpnea and salivation.

2. Suppression of perspiration and salivation by atropine or antidiaphoresis by agaric acid results, during exposure to heat, in still higher body temperatures and is usually followed by death (cats and rabbits).

3. Active sweat glands of cats (glomeruli as well as the proximal portions of the ducts) prevent neutral fuchsin methylene blue from turning blue in the presence of molecular oxygen, while inactive tissues become stained. This procedure may be considered as a qualitative test of function of the sweating apparatus.

4. The adnexa of the sweat glands, fibrillar bodies (myo-epithelial elements), shrink during sweating and expand again during anhidrosis. This volume decrease appears to be due to loss of water which may contribute to the initial outflow of sweat.

5. During sweating, the portion of the duct in the stratum corneum changes from a tight spiral to a loosely convoluted tube. The presence of ampullae in this portion of the duct at irregularly spaced intervals suggests that the outflow of sweat proceeds in a succession of droplets.

6. The mucous glands in the skin of frogs exhibit the same reactivity to specific pharmacological agents as the sweating apparatus of mammals. Atropine and agaric acid inhibit mucous secretion upon systemic, and aluminum chloride upon local, administration.

LITERATURE CITED

- BEST, C. H., AND N. B. TAYLOR 1945 The Physiological Basis of Medical Practice, 4th Edition, p. 626-627. The Williams and Wilkins Co.
- BUNTING, H., G. B. WISLOCKI AND E. W. DEMPSEY 1948 The chemical histology of human eccrine and apocrine sweat glands. *Anat. Rec.*, 100: 61-78.
- COON, J. M., AND S. ROTHMAN 1941 The sweat response to drugs with nicotine-like action. *J. Pharm. and Exper. Therap.*, 73: 1-11.
- FENN, W. O. 1936 The role of spaces in the osmotic equilibrium of frog muscles in hypotonic and hypertonic solutions. *J. Cell. and Comp. Physiol.*, 9: 93-103.
- FISHER, E. 1936 Volume changes of smooth muscle of *Phascosoma* during activity. *Proc. Soc. Exp. Biol. and Med.*, 34: 707-708.
- FISCHER, E., AND E. MOROCZ 1941 Volume diminution and potassium ionization in muscle during activity. *Enzymol.*, 9: 133-134.
- GURNEY, R., AND I. L. BUNNELL 1942 A study of the reflex mechanism of sweating in the human being; effects of anesthesia and sympathectomy. *J. Clin. Invest.*, 21: 269-274.
- HOEPKE, H. 1927 Die Haut. v. Möllendorff's Hand. d. micros. Anat. d. Mensch. Bd. III, Teil I: 61 Julius Springer, Berlin.
- HEYNOLD, H. 1874 Über die Knäueldrüsen des Menschen. *Virchow's Arch. f. Path. Anat. u. Physiol.*, 61: 77-90.
- HUNTER, F. R., AND A. K. PARPART 1938 Solvent water in frog muscle. *J. Cell. and Comp. Physiol.*, 12: 309-312.
- HYMAN, L. H. 1942 Comparative Vertebrate Anatomy, 2nd Edition, p. 81-82. University of Chicago Press.
- KOPPANYI, T. 1945 Hexadienol, a locally acting diaphoretic and a new diagnostic agent. *J. Am. Pharm. Assoc.*, 34: 221-224.
- LIST, C. F., AND M. M. PEET 1938 Sweat secretion in man. I. *Arch. Neur. and Psych.*, 39: 1228-1237.
- 1938 Sweat secretion in man. IV. *Arch. Neur. and Psych.*, 40: 443-470.
- MAXIMOW, A. AND W. BLOOM 1948 A Textbook of Histology. 5th Edition: p. 177. W. B. Saunders Co.
- MEYERHOF, O. 1947 The main chemical phase of the recovery of muscle. *Ann. N. Y. Acad. Sci.*, 47: 815-834.
- MEYERHOF, O., AND W. MÖHLE 1933 Über die Volumenschwankung des Muskel in Zusammenhang mit dem Chemismus der Kontraktion. III. Über die Volumänderung bei Chemische Vorgängen. *Biochem. Zeitschr.*, 261: 262-266.
- OLIVET, I., AND E. TH. NAUCK 1930 Histologische Untersuchungen der Groben Achselhöhlen-Schweissdrüsen nach Einwirkung von Pilocarpin Atropin und Adrenalin. *Zeitschr. f. d. ges. Exper. Med.*, 71: 786-799.
- RENAUT, J. 1894 Dispositif Anatomique et Mechanisme de l'Excretion des Glandes Sudoripares. *Ann. de Dermatol. et Syph.*, 5: 1101-1104.
- RING, J. R., AND W. C. RANDALL 1947 The distribution and histological structure of sweat glands in the albino rat and their response to prolonged nervous stimulation. *Anat. Rec.*, 99: 7-20.

- SHELDON, W. F. 1941 The myoepithelium in sweat gland tumors. *Arch. Path.*, 31: 326-337.
- SHELLEY, W. B., P. N. HORVATH AND S. M. HORVATH 1948 Inhibition of sweating by means of iontophoresis. *Fed. Proc.*, 7: 114.
- SINGH, I. 1944 The swelling of untreated muscle produced by certain ions and its relation of permeability, excitability, absorption and secretion. *Proc. Ind. Acad. Sci., Sec. B*, 20: 209-218.
- SOLLMANN, T. 1948 *A Manual of Pharmacology*. 7th Edition, p. 35 and p. 325. W. B. Saunders Co.
- SPERLING, F. 1946 Detection of oxidation-reduction by alkaline solutions of methylene blue and orcein. *Fed. Proc.*, 5: 205.
- SPERLING, F., AND T. KOPPANYI 1947 Pharmacomorphology of the sweat glands of the paw of the cat. *Anat. Rec. (Suppl.)*, 26: 54.
- 1947 Pharmacomorphology of the sweat glands of the cat's paw. *Anat. Rec. (Suppl.)*, 29: 68.
- SPERLING, F., AND T. O. KING 1947 Methylene blue as an indicator of diaphoresis in the cat. *Anat. Rec. (Suppl.)*, 29: 67.
- STEINBACH, H. B. 1947 Intracellular inorganic ions and muscle action. *Ann. N. Y. Acad. Sci.*, 47: 849-874.
- WAY, S. C., AND A. MEMMESHEIMER 1936 The sudoriparous glands. I. The eccrine glands. *Arch. Dermatol. and Syph.*, 34: 797-808.

PLATE 1

EXPLANATION OF FIGURES

All figures are from preparations of the cat's paw.

1 Unstimulated, non-sweating glands, showing myoepithelial element. Note the numerous processes extending from each end of the central portion of the element. Fixed with Helly's fluid and stained with phosphotungstic acid hematoxylin. $\times 450$.

2 Same, $\times 900$, showing the fibrillar structure of the element.

3 Myoepithelial elements during profuse sweating induced by pilocarpine. Note the diminution in size, and the reduction in the number of processes present. Fixed with Helly's fluid and stained with phosphotungstic acid hematoxylin. $\times 450$.

4 Myoepithelial element in a gland in which profuse sweating has been stopped by intra-arterial injection of ferric chloride. Note the spindle shape of the element. It appears to be smaller than the element in figure 3. Fixed with Helly's fluid and stained with phosphotungstic acid hematoxylin. $\times 450$.

5 Duct of an unstimulated, non-sweating gland passing through the stratum corneum. Note the spiral form. Unfixed frozen section; unstained. $\times 450$.

6 The duct as seen during profuse sweating. Ampulla marked by arrow. Stained with eosin and methylene blue. Unfixed frozen section. $\times 450$.

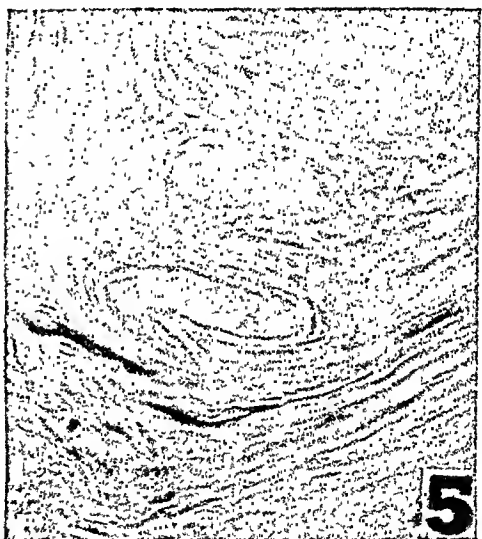
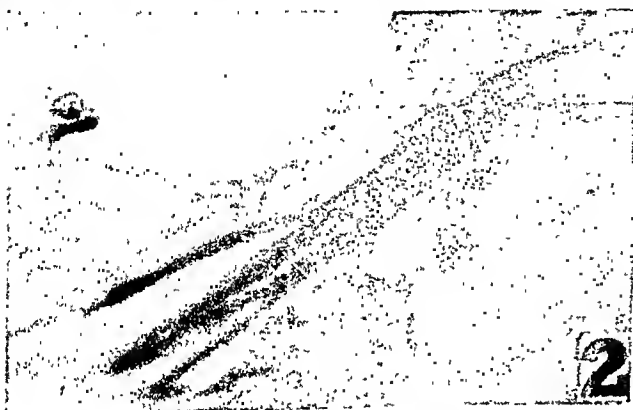
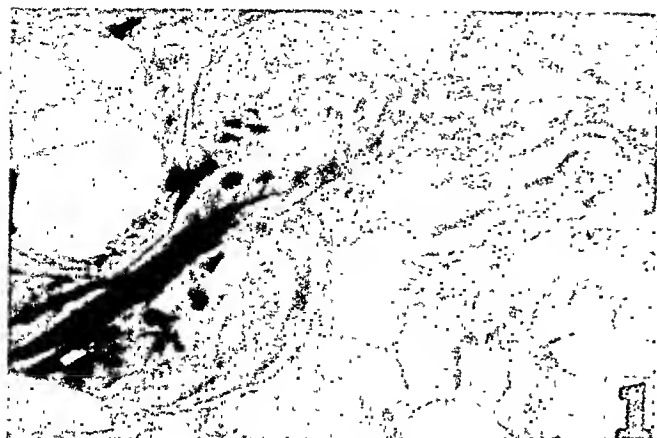


PLATE 2

EXPLANATION OF FIGURES

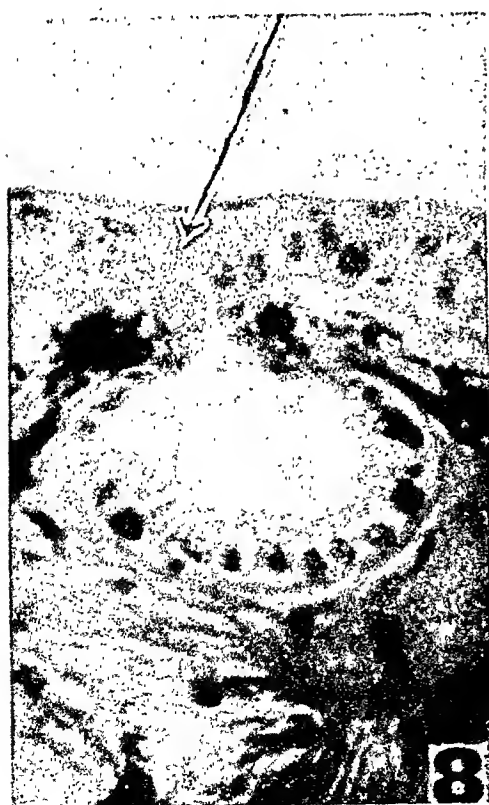
In each figure the duct is marked by an arrow.

7 Mucous gland. Note the large amount of mucus present and the lack of cellular outlines. Stained with Harris' hematoxylin and eosin.

8 Appearance of mucous gland of frog skin after the intramuscular injection of agaric acid. Note the diminution in amount of mucus and the appearance of cellular outlines. Stained with Harris' hematoxylin and eosin.

9 Mucous gland after the intramuscular administration of atropine. The appearance is similar to that seen after administration of agaric acid.

10 Note the greatly diminished size of the epithelium and the few strands of mucus remaining in the gland after application of alum.



THE SEBACEOUS GLANDS OF THE HAMSTER

II. SOME CYTOCHEMICAL STUDIES IN NORMAL AND EXPERIMENTAL ANIMALS ¹

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SIXTEEN FIGURES

In another paper (Hamilton and Montagna, '49) we have described the morphology of the sebaceous glands of the skin of the hamster under normal and under certain experimental conditions. Special study was made of the glands of the pigmented costovertebral spot in normal adult males and females, in ovariectomized females, and in ovariectomized females which were injected daily with testosterone propionate for different periods. This paper reports a series of cytochemical observations on the sebaceous glands of animals under comparable conditions.

MATERIAL AND METHODS

For the study of sebaceous glands in the costovertebral pigmented spot of hamster, we removed a piece of skin one inch in length. These large samples contained not only the whole spot but also enough ordinary skin for the study of the sebaceous glands elsewhere. Samples of skin from the flank and belly were also collected.

We used 11 normal adult males and approximately 30 experimental animals. Immature females, stated to be three weeks old, were ovariectomized when they were received

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from the breeding laboratory. Beginning three weeks after ovariectomy some of these animals were given daily subcutaneous injections of 2 mg of testosterone propionate in 0.05 cm³ of peanut oil, others were left untreated to be used as controls. Animals receiving androgenic injections were killed one day, two, three, 4 5, 6, 8, 10, 15 and 25 days after the beginning of treatment. Comparable untreated ovariectomized control animals were killed at the same intervals.

For the study of lipids, tissues were fixed in 10% neutral formalin, and formal-calcium-cadmium (Baker, '44). Frozen sections were: (a) stained with Sudan IV and Sudan black B for total lipids; (b) stained with Nile blue sulfate for the detection neutral fats (Smith, '08, '11; Cain, '47a); (c) tested with the Smith-Dietrich method, and the acid-hematein, and pyridine extraction tests of Baker ('46, '47) to determine the presence of phospholipids; (d) studied unstained under polarized light and near-ultraviolet light for anisotropy and autofluorescence; (e) treated with modifications of the Liebermann-Burchard test (from Lison, '36) for the presence of cholesterol and its esters; (f) stained with the Schiff reagent and 2, 4-dinitrophenylhydrazine for the presence of plasmal (Albert and Leblond, '46).

For the study of the Golgi substance tissues from a few males were prepared by the DaFano method, or fixed in a mixture of 1 ml 2% osmic acid, 4 ml 1% chromic acid, and one drop of glacial acetic acid, then post-osmicated for 5 days at 37°C. (Worley, '46).

For the demonstration of phosphatases and lipase, tissues were fixed in chilled acetone, cleared in benzene, and imbedded in paraffin. Alkaline phosphatase was revealed by the method of Gomori ('41a); acid phosphatase by the procedure of Gomori ('41b), and of Wolf et al. ('43); and lipase by the method of Gomori ('46).

For cytoplasmic basophilia, Zenker-formol fixed tissues were stained with the standard eosin-methylene blue method. Control sections were incubated in 0.1% of ribonuclease buffered to pH 6.7 before staining.

RESULTS

General features. Hamsters possess midway on their backs a pair of clearly outlined black spots. These specialized cutaneous areas are characterized by abundant melanin, very large sebaceous glands and coarse black hairs. The spots are larger in normal males than in females or in castrate males, and their gross size in females and immature animals can be increased upon stimulation by male hormone.

The morphology of the sebaceous glands in the costovertebral spot of the hamster has been described by Hamilton and Montagna ('49) in normal males and females, castrated females, and castrated females stimulated with androgenic treatment. Briefly stated, the glands of castrated females were small and generally inactive. Those of castrated females injected daily with 2 mg of testosterone propionate showed a progressive and marked increase in the size and activity of the sebaceous glands of the pigmented costovertebral spot, and to a pronounced, but lesser extent, in the glands of the general skin of the body. After 25 days of androgenic stimulation, the glands were as large as those of normal males and in a more active functional state. After 60 days they had grown still larger and were correspondingly active.

LIPID SUBSTANCES

The present account deals with a study of the lipid substances found in the sebaceous glands of the normal and experimental animals. Separate descriptions are given in each section for the glands of the general skin and for the larger glands of the spot. The spot appears to be a male secondary sex character (see Kupperman, '44) and the reactions of its sebaceous glands to androgens are more profound than those of the sebaceous glands in other regions of the skin.

1. Sudan IV and Sudan black

Normal adult male. Sudan IV demonstrates abundant lipid in the sebaceous glands of the spot. On the basis of the

distribution of sudanophilic droplets the acinar cells were disposed into 4 layers (figs. 1 and 2). Progressing from the periphery of the acinus to the center they were: *A*. A layer of small cells, one or more cells in thickness, containing small lipid droplets at times but often devoid of sudanophilic lipids. *B*. A band, two or more cells in thickness, in which the sebaceous cells contained large sudanophilic globules that stained a brilliant brick-red. This region was distinctive in well-stained preparations. *C*. A third band, usually one cell in thickness, where the lipid globules stained poorly with Sudan IV, although they were as large as those found in the intensely colored layer peripheral to it. *D*. The center of the acini was occupied by sebum and degenerating sebaceous cells; these were richly sudanophilic. Some of the lipids were still in the form of discrete globules, but others formed large amorphous masses mingled with cellular debris. This concentric stratification was more evident in the larger acini than in the smaller ones, and not all of the large ones displayed it with equal clarity.

In the peripheral sudanophilic band *B* the large cells contained in their cytoplasm many discrete lipid spherules that appeared like clusters of grapes displacing the nucleus peripherally. In those cells with few lipid globules, these were always found close to the nucleus. In cells with the most advanced degree of sebaceous changes, many of the globules had coalesced.

The glands of the general skin were much smaller than those of the pigmented spot. They contained abundant lipids, but Sudan IV did not reveal the lamellation described above.

The peripheral portions of hair follicles were usually filled with sebum. Tufts of hairs emerged upon the skin bathed in sudanophilic sebum. Sudanophilic substances were also found along the shafts of hairs deep to the entrance of the glands into the follicles.

The stratum corneum of the surface epidermis in the spot and in the general skin was deeply and equally sudanophilic.

Staining with Sudan black or with oil-blue N revealed a more extensive distribution of lipids. The glands stained blue-black or blue respectively. With both these dyes the distinct lipid globules as well as many cytoplasmic inclusions stained clearly. Those cytoplasmic sudanophilic particles which did not stain with Sudan IV and oil-blue N but which stained clearly with Sudan black appeared to correspond to substances which stained with acid hematein (*vide infra*). The peripheral cells of the acini contained distinct sudanophilic particles, especially around the nucleus. The stratification noted with Sudan IV and with oil-blue N was not marked in glands stained with Sudan black.

When the frozen sections of tissues that were fixed in formal-calcium-cadmium were placed in warm alcohol or in acetone, or when they were dehydrated in alcohols and cleared in xylol and then brought back to 70% alcohol, and subsequently stained with Sudan black, much of the sebum and the degenerating sebaceous cells were still sudanophilic, but the rest of the gland was not. Probably the solubility of the lipids in the sebum and in the degenerating sebaceous cells was different from that of lipids in less mature sebaceous cells (*cf.* Montagna and Noback, '46, in the preputial gland).

Ovariectomized females. The sebaceous glands of the pigmented spot in adult castrated females (fig. 3) were much smaller than those of adult normal males (fig. 2). In rare instances a few acini showed some lipids in the sebaceous cells, and in the sebum. Most acini contained a minimal amount of stainable lipids limited to the central cells which were about to undergo sebaceous degeneration (fig. 3). Many acini contained practically no demonstrable lipids.

The sebaceous acini in the general skin were very small. Most of them possessed a small core of slightly sudanophilic sebum and minute lipid globules in the cells.

The thin stratum corneum over the spot and elsewhere in the skin was sudanophilic but to a lesser degree than in normal adult males.

Ovariectomized females treated with androgen. When stained with Sudan IV and other oil-soluble dyes, the glands of the pigmented spot of ovariectomized animals which had been treated with testosterone propionate for one day were practically similar to those of untreated castrates. The central cells of the larger acini, however, showed a slight increase of lipid accumulation over the untreated castrates.

In animals treated for two, three, and 4 days there was a progressive increase in lipid droplets in the sebaceous cells. In animals treated 5 days the sebaceous cells were full of large lipid droplets (fig. 4). The glands of animals treated 10 days (fig. 5) were almost as large as those of normal males (fig. 2). The distribution of sudanophilic globules in the sebaceous cells appeared like those of normal males; there was even a suggestion of concentric stratification. In animals treated for longer periods the glands became still larger.

The sebaceous glands in the general body skin also showed a progressive accumulation of lipid substances. In animals stimulated with androgenic treatment beyond 10 days the sebaceous cells exhibited a maximal accumulation of sudanophilic droplets. Although abundant in individual cells these droplets were never as large as those in the glands of the costovertebral pigmented spot.

The stratum corneum of the skin in the spot, as well as elsewhere in the body, became increasingly thicker and more sudanophilic with continued androgenic treatment.

Nile blue sulfate

The Nile blue sulfate technique stains triglycerides a rose color (Smith, '11), and other materials, not necessarily lipids, in varying shades of blue. Cain ('47a) reports that a warm solution of 0.05% Nile blue sulfate possibly stains fatty acids selectively blue.

Normal adult males. When this technique was applied to skin of the pigmented spot, the sebaceous acini displayed a concentric stratification of colors.

Progressing centripetally: *A.* The peripheral cells stained homogenously light blue. *B.* A band, usually two cells thick, in which the large lipid droplets stained a pale rose color (compare with description of glands stained with Sudan IV and oil-blue N). *C.* A band usually one cell thick and whose cytoplasm stained a deep blue while the lipid droplets stained a very pale pink. *D.* In the mature, and in the degenerating cells the lipid droplets stained a bright pink. The newly formed sebum in the center of the acini was usually purplish; in the ducts it acquired a lilac color, and the old sebum in dilated ducts, or in the orifices of hair follicles, was pink to red.

Sections which had been immersed for 30 minutes in warm alcohol or in acetone, previous to staining with Nile blue, revealed neither rose-colored nor blue-staining lipid droplets.

In the small sebaceous glands elsewhere in the skin a narrow peripheral band of cells was stained blue with Nile blue sulfate. The other acinar cells stained a dark pink color. The sebum was a mottled purple.

In most instances the thick stratum corneum over the spot and elsewhere in the skin was a bright pink.

Ovariectomized females. The small glands in the spot of castrated females were stained mostly blue, but a few cells contained purplish droplets. The small amount of sebum was usually a deep purple.

The glands of the general skin were stained blue throughout. The sebum and the degenerating cells were purplish.

The thin stratum corneum everywhere stained light pink.

Ovariectomized females treated with androgen. The glands of the pigmented spot of castrated females injected with androgen for only one day, when stained with Nile blue sulfate, appeared similar to those of control untreated castrates. The centers of all the acini contained traces of a lilac color. The rest of the glandular substance stained blue. In the glands of animals treated two, 4, 5, 6, and 8 days with androgen there was a gradual increase of pink color from the center of the acini toward the periphery. The

glands of animals treated 10 days, except for the blue color in the periphery, were lilac to purple. After 10 days of androgenic treatment some acini displayed a vague stratification of colors such as described in the glands of males. Even in animals treated for 25 days, concentric strata were not as clearly indicated as in normal adult males.

After androgenic treatment there was a larger amount of sebum, the ducts become increasingly dilated, and the center of the acini underwent more active degeneration. The glands of animals treated with testosterone propionate for 25 days were larger than those of normal adult males, and sebaceous activity appeared greater. The sebum was more pink than that of adult males, suggesting, perhaps a greater amount of triglycerides and a smaller amount of blue-staining substances.

The sebaceous glands outside of the spot also showed a progressive increase of triglycerides with continued androgenic treatment. In animals treated 8 days or longer the bulk of the acinus, with the exception of a narrow peripheral band, stained pink to lilac.

With androgenic treatment the stratum corneum everywhere became thicker and more intensely rose colored.

Birefringent lipids

Normal adult males. Under polarized light the glands of the pigmented spot showed birefringent substances in the large degenerating acinar cells and in the sebum (fig. 8). The anisotropic particles were either dust-like or acicular. The peripheral acinar cells contained no birefringent particles. Occasionally, some birefringent particles were distributed in the peripheral sudanophilic band *B*. The lipid droplets in these acinar cells were isotropic but each droplet (1 to 3 μ in diameter) was encircled by very small birefringent crystals. The most brilliant birefringence was found in the sebum. In the core of the gland where the newly-formed sebum contained recognizable cellular fragments, it appeared mottled.

Toward the outer portion of the duct it became progressively brighter and showed a brilliant yellowish-white birefringence.

Immersion of sections in acetone only diminished the anisotropy of the sebum. In the sebaceous cells it was quickly abolished. The anisotropy of the stratum corneum of the skin was destroyed only after immersion for 48 hours in acetone or warm alcohol.

Ovariectomized females. In most instances the acinar cells of the glands in the spot of control animals did not possess birefringent crystals, but a few anisotropic crystals were found occasionally in those cells which were undergoing sebaceous degeneration.

The small amount of sebum was usually brilliantly birefringent whether it was found in the ducts or in the center of the acini. This differs from the condition found in the adult male where the sebum within the gland was always weakly birefringent.

The stratum corneum over the spot and elsewhere in the skin appeared as a very thin birefringent band.

Ovariectomized females treated with androgen. In the glands of the spot of animals injected with androgen for one day, the anisotropy was essentially similar to that of castrated controls. Comparable to findings obtained with other tests, in animals injected for two, three, 4, 5, 6, 8, 10, and 25 days, there was a gradual increase in the amount of birefringent crystals in the glands. In animals injected 10 to 25 days the distribution of anisotropy was even more extensive than that of the normal adult male.

The glands of the general body skin, in animals injected for 10 to 25 days, were large and active. The abundant sebum and the degenerating sebaceous cells showed anisotropic crystals.

The birefringence of the stratum corneum over the spot and elsewhere in the skin also increased with continued injection. At 10 days it was as extensive as that of normal adult males.

Fluorescence

Normal adult males. The sebaceous glands of the pigmented spot fluoresced with a dull, barely visible, gray light at the periphery of the acinus. The degenerating sebaceous cells showed a grayish-yellow light of medium intensity; the newly formed sebum emitted a yellow light of medium intensity, and the older sebum in the enlarged sebaceous pockets near the surface of the skin show a bright yellow to orange light.

The skin glands outside the spot showed a very small amount of dull grayish-yellow fluorescence in the acinus, but the sebum was bright yellow.

The stratum corneum emitted a yellow light which was more intense over the spot than elsewhere in the skin.

Ovariectomized females. In castrated untreated females most of the acini of the glands of the spot had a dull gray color of very low intensity. An occasional acinus showed a dull yellowish hue especially toward the center. Sebum, which was present in small amounts in some acini, fluoresced with a yellow color of medium intensity, and near the surface of the skin it emitted a bright yellow light. The glands of the general body skin showed no distinctive fluorescence.

Ovariectomized females treated with androgen. In animals injected with androgen for one day the glands of the spot showed no noticeable difference from those of control castrates. The fluorescence increased gradually in the animals injected two, three, 4, 5, 6, 8, and 10 days. In animals treated for 10 days or longer the fluorescence of the glands of the spot as well as those of the general skin was like that of normal males.

Fischler technique

Lillie's ('48) modification of Fischler's ('04) test for fatty acids is based on the principle that in tissues fixed in 10% formalin which has been saturated with calcium salicylate, fatty acids form insoluble calcium soaps (see Lillie for details of solubility tests). After mordanting with cupric

acetate, sections are laked with hematoxylin and then differentiated in Weigert's borax-ferricyanide mixture. Those substances which resist differentiation may represent fatty acids.

Normal adult males. In the sebaceous glands in the spot of adult male hamsters, with the exception of the peripheral ones, the acinar cells showed blue to black granulation. The Fischler-positive substances were either in the form of granules or formed a film over most of the large lipid globules. The cells undergoing sebaceous degeneration were usually completely dark blue to black (the discrete lipid spherules remained colorless).

The newly formed sebum was always dark blue, but the older sebum in the sebaceous ducts near the orifices was usually colorless except for a blackish mottling. When sections treated with this method were further stained with Nile blue sulfate, the Fischler-negative old sebum became pink.

The stratum corneum of the large ducts and of the skin always stained blue to black.

Ovariectomized females. In the glands of the spot as well as elsewhere in the skin only the sebum stained. The acinar cells showed no evidence of Fischler-positive substances.

Ovariectomized females treated with androgen. The glands of the spot began to show blue-black granulation in the sebaceous cells after three days of treatment. The reaction progressed until the 10th day when the distribution of the Fischler-positive substances resembled that in the glands of adult males. The staining property of the sebum was similar to that described in the glands of normal males.

Phospholipids

After the use of the Smith-Dietrich method, a positive black to blue stain (fig. 6) is considered to be somewhat specific for phospholipids, if one can exclude the presence of cholesterides (Lison, '36; Baker, '44). Baker's ('46, '47) acid-hematein test is more sensitive than the Smith-Dietrich

test and can be controlled with the pyridine extraction test. Baker's methods, handled with caution, are believed to reveal phospholipids in tissues (Cain, '47b).

Both the Smith-Dietrich method and the acid-hematein test (in combination with the pyridine-extraction test) were applied to the sebaceous glands of normal male hamsters. The acid-hematein test gave the best results; these are described here.

Normal adult males. The peripheral band of the acini, one or two cells in thickness, showed dust-like bluish granules that could be seen clearly only at high magnifications. The cytoplasm in the layer of cells next removed from the periphery of the acinus was filled with granules and rodlets which stained blue to black. In sebaceous cells fully engorged with lipid droplets (fig. 9) the filamentous cytoplasm between the lipid droplets also contained dark granules (as in the sebaceous glands of the dog described by Montagna and Parks, '48). The cells located more centrally in the acinus possessed coarser granules surrounding the unstained lipid droplets, giving these cells a darker appearance than the more peripheral ones. The cytoplasm of an occasional central acinar cells was completely blue-black and showed no granulation, but the large lipid spherules remained colorless. These cells represented foci where sebaceous degeneration was about to begin. The sebum at the apex of the glands was also blue-black but it was fenestrated by colorless globules or streaks. In sections stained with acid hematein and subsequently treated with Nile blue sulfate the colorless areas just mentioned became pale pink.

Sections which had been extracted with pyridine previous to staining with acid-hematein (Baker, '46), showed no blue-black cytoplasmic inclusions. The degenerating sebaceous cells and the sebum, however, stained brown to blue. The color was not as intense as that observed in tissues not extracted with pyridine. This persistent dark stain then cannot represent lipine, but rather some protein-containing substances which also stain with acid-hematein (Baker, '46).

This does not rule out the presence of some lipine in the degenerating sebaceous cells and in the sebum.

Ovariectomized females. In the glands of castrated females, acid-hematein revealed little of note in the sebaceous cells. Vague dust-like granules were found in the cytoplasm around the nuclei. The degenerating cells and the sebum were blackened.

Ovariectomized females treated with androgen. The glands of castrated females stimulated with androgens showed an increase of acid-hematein-positive particles. On the 10th day of treatment the glands were practically indistinguishable from those of normal adult males.

*DaFano method for Golgi apparatus and
osmiophilic bodies*

Only tissues from adult males were treated with the Da Fano method. In the glands of the spot, the cytoplasm of sebaceous cells contained black particles, whose distribution resembled that of the acid-hematein-positive particles (figs. 9 and 10). In the mature sebaceous cells, the fine strands of cytoplasm between the lipid droplets stained black. In less mature cells, especially in those toward the periphery of the acini, only fine black particles were observed; the rest of the cytoplasm remained colorless.

Skin from the spot of adult males was fixed in a chromic-acid-osmium tetroxide solution with a trace of acetic acid, and post-osmicated for 5 days. In the pigmented spot, the peripheral sebaceous cells contained either no osmiophil particles, or small blackened granules around the nucleus. The larger peripheral cells (fig. 7), comparable to band *B*, possessed coarse black granules around the nucleus. Inside this band of cells there was a layer, two or more cells deep (comparable to band *C*, which, it will be recalled, is weakly sudanophilic) in which all of the cells contained large osmiophilic lipid globules only around the nucleus. Although these cells contained other large lipid spherules in the cytoplasm,

these remained colorless. The osmiophilic globules were often arranged in a ring around the nucleus. In the mature sebaceous cells and in those undergoing sebaceous breakdown the lipid droplets were osmiophobic but they were surrounded by a delicate black ring. This phenomenon was more pronounced around the nucleus but practically all of the droplets were enclosed in an osmiophilic film. The cytoplasmic reticulum between the lipid vacuoles was gray to black (cf. with acid-hematein test). The sebum in the center of the glands, which contained recognizable cellular debris, was usually gray. The older sebum in the sebaceous ducts became gradually darker and was black near the exits.

In the sebaceous glands of the general body skin the osmiophilic bodies were different from those of the gland of the pigmented spot. The lipid globules remained colorless. In the cytoplasm between the vacuoles, coarse black or brown granules were abundant. These osmiophilic bodies were most abundant in the mature and in the degenerating sebaceous cells. The sebum resembled that in the glands in the spot.

Modified Liebermann-Burchard tests

Two modifications (Schultz and Romieu, from Lison, '36) of the Liebermann-Burchard tests for cholesterol, or cholesterol-like substances, and their esters, have been applied to the sebaceous glands of the spot. There was a weakly positive reaction in the glands of normal males, castrated control females and castrated females injected with testosterone propionate. In the sebum of the glands of normal males and of castrated females treated with androgen for 10 days or longer, there was a progression of color changes (brown to lilac to red, fading to greenish-blue) which indicates the presence of cholesterides. Since the reaction was not very strong it must be assumed that the amount of cholesterides present in the sebum is small (control section of suprarenal gave strong positive results).

Plasmal reactions

The plasmal reaction, which may demonstrate aldehydes of fatty acids, follows the use of the Schiff reagent, or the 2, 4-dinitrophenylhydrazine reaction (Albert and Leblond, '46; Gomori, '42). These tests gave similar results in the sebaceous glands of the hamster. The glandular substance of all animals, normal males, castrated females, and castrated females injected with androgens, always showed a mild reaction. Stronger reactions were noted in the sebum, in normal males and in experimental animals injected for 10 days or longer. Staining of greater intensity was found in the old sebum near the orifice of the ducts.

ENZYMES

Alkaline phosphatase. The sebaceous glands of all the hamsters investigated, normal adult males, control castrated females, and castrated females treated with testosterone propionate, showed traces of alkaline phosphatase activity. A pale gray cast was often found in the acinar cells and in the nuclei, but the reaction was never strong.

The epidermis sometimes became brownish. Tufts of connective tissue in the dermis were occasionally blackened.

Acid phosphatase. There was strong acid phosphatase activity in the sebaceous glands of adult males, and of experimental animals (figs. 14, 15, 16). In the peripheral acinar cells enzyme activity was restricted to the nuclei. The other acinar cells, in contrast, stained intensely dark brown. The intensity of the reaction was strong throughout the acinus, but in parts undergoing cellular degeneration it was weaker. The newly formed sebum in the center of the acinus was always black. The older sebum in the ducts was brown with mottling of black.

The sebaceous glands in the general skin, like glands of the spot, showed acid phosphatase activity in the acinar cells and sebum.

Lipase. In the glands of adult male hamsters lipase activity could be detected in the form of coarse, brown granules

scattered sparsely through the acinar cells (figs. 12, 13). In those cells which were undergoing sebaceous degeneration, enzymatic activity appeared to be maximal. The cytoplasm of these cells was loaded with brown to black granules. In those immature acini which did not contain sebum, the central cells only showed conspicuous lipase activity, whereas the rest of the acinar cells contained only a few brown granules.

The sebum, new and old, exhibited abundant lipase activity. It stained dark brown to black.

The glands of ovariectomized females showed enzyme activity primarily in the sebum, as in normal animals (fig. 11). Lipase activity was scanty or absent in the acinar substance.

In the glands of ovariectomized females injected with testosterone propionate, lipase activity increased concomitantly with the augmented activity of the glands. In animals injected 25 days, enzyme activity was abundant, perhaps even more so than in normal males.

The glands of the general body skin showed lipase activity principally in the sebum.

Dense brown granulation was observed also in the stratum corneum. It was more abundant in the skin of normal males and androgen-treated animals than in ovariectomized females.

BASOPHILIA

When the sebaceous glands of adult males and of androgen-treated ovariectomized female hamsters were stained with eosin-methylene blue the peripheral acinar cells stained a deep blue. The basophilia diminished gradually toward the center of the acinus and was least abundant in those cells whose cytoplasm was engorged with lipid globules. The cells which were undergoing sebaceous degeneration were always strikingly eosinophilic as was also the sebum. Sections incubated for three hours at 60°C. in a 0.1% solution of ribonuclease buffered to pH 6.7 and stained subsequently with eosin-methylene blue, or toluidin blue, no longer possessed

cytoplasmic basophilia, although the basophilia of the nuclei remained virtually unchanged. After incubation in ribonuclease, the nucleoli showed an acidophilic reaction.

Caspersson et al. ('41), and others, have presented evidence that the cytoplasmic basophilia which disappears after digestion by ribonuclease is due to the presence of ribonucleoproteins.

DISCUSSION

The large glands of the spots of normal males display an interesting distribution of lipid particles when they are stained with Sudan IV and oil blue N. Proceeding from the periphery to the center, 4 zones can be recognized in these glands: *A*, a band of small cells which sometimes contain few small sudanophilic bodies; *B*, a band whose cells contain large sudanophilic spherules around the nucleus; *C*, a band whose cells contain detectable lipid globules around the nucleus but these globules are practically not sudanophilic, and *D*, the mature sebaceous cells whose lipid droplets stain clearly. In sections stained with Sudan black this stratification is much less distinct and the lipids are more intensely stained. The glandular stratification is pronounced in preparations stained with Nile blue sulfate. In such preparations the larger lipid droplets usually stain with the pink oxazone, with the exception of the perinuclear lipid droplets in the cells in band *C*, which are not stained with the pink oxazone and perhaps do not contain triglycerides. In the sebaceous cells of band *C*, the lipid droplets in the cytoplasm, away from the nucleus, are weakly sudanophilic and stain light pink with oxazone. Preparations with osmic acid show an interesting correlation with these observations. A stratification of the lipid substances is encountered again. The perinuclear lipid droplets in bands *B* and *C*, and principally in band *C* are osmiophilic and become completely blackened (fig. 7). The lipids in the cytoplasm distant from the nucleus are usually osmiophobic. In the mature sebaceous cells in the center of the acini the lipid spherules are osmiophobic but

they are always enclosed in a delicate black ring. Although osmic acid is capricious in its reaction, and is notoriously non-specific, there is some consistency in its behavior in these glands: those lipids which stain weakly or not at all with oil-soluble dyes (except with Sudan black) are osmiophilic, but those which stain readily with them are osmiophobic. The perinuclear sudanophilic globules in band *B* are an exception to this statement. The lipid globules in the same gland or in the same cell, then, do not react alike to Sudan IV, Nile blue and osmic acid.

These observations, perhaps, throw some light upon the progression of lipid synthesis in these glands. Lipid storage in the sebaceous glands is more abundant in the central cells and it decreases progressively toward the peripheral cells. The central degenerating sebaceous cells are fully laden with lipid droplets even in glands whose peripheral cells are small and practically non-lipoidal. Lipid synthesis and storage within the individual cells appears to begin around the nucleus, perhaps in association with the Golgi element. One might surmise that chronologically, the lipids in the central acinar cells are older than those in the peripheral cells and that those in an individual cell in the peripheral cytoplasm are older than the perinuclear globules. If the perinuclear osmiophilic lipid droplets actually represent newly accumulated lipids then they must contain a substance which reduces osmic acid. Whatever this substance complex may be, it appears to be lost or changed in the older lipid droplets. It is puzzling that the older lipid globules, which appear to contain triglycerides, do not reduce osmic acid, although adipose tissue in the same sections is always blackened. Bowen ('26) found that in the cells of the white inguinal glands of the rabbit, the lipid globules were osmiophilic in some cells and osmiophobic in others. In the Meibomian glands of the cat he found that the lipid secretion-droplets did not blacken when sections were treated with osmic acid for the study of Golgi bodies. Ludford ('26) describes, in the sebaceous glands of the mouse, a progressive blackening of the lipid

droplets associated with the maturation of the cells. At present we do not understand these discrepancies; further work may clarify them.

In the sebaceous cells the particles revealed by Baker's acid-hematein test appear to represent fragments of Golgi bodies (in agreement with Ludford, '25; and Bowen, '26, '29). Perhaps they also represent mitochondria (in agreement with Nicolas et al., '14). In the younger cells they are oriented around the nucleus; in more mature sebaceous cells they surround the lipid globules. In preparations for the Golgi apparatus with the method of DaFano, the picture obtained is virtually similar to this (fig. 10).

The sebum of hamsters contains glycerides, cholesterides, phospholipids, plasmalogens and traces of Fischler-positive substances (fatty acids?). The distribution of these substances in the acinar cells was described above and needs no further comment. The nature of the newly-formed sebum differs from that of older sebum, although the sebum is uniformly sudanophilic.

The Nile blue sulphate test shows that the degenerating sebaceous cells as well as the newly-formed sebum closely associated with them, contained comparably small amounts of pink-staining substances (triglycerides). The sebum in the sebaceous ducts near the surface of the skin, however, stains a clear pink color with negligible amounts of blue staining substance. Sections in which the duct was cut longitudinally showed the gradual change of color from purple in the new sebum to rose in the old sebum. This suggests that perhaps in the maturation of the sebum there is either a gradual increase in its content of triglycerides, or that substances which stain with the blue oxazine base diminish in the old sebum.

In the sebum, the distribution of plasmal closely follows that of the triglycerides.

The new sebum is Fischler-positive, staining dark blue to black. Nearer the exit of the duct, however, the color becomes paler and the sebum plug at the orifice of the duct is practically colorless. If this test were really specific for fatty

acids (Fischler, '04), then it would appear that the sebum, which begins with a rich content of fatty acids, ends by containing practically none.

With the Smith-Dietrich method and Baker's ('46, '47) acid-hematein test for phospholipids, the new sebum stains black, the old sebum usually blue. Phospholipids, then, may also be less abundant in the old sebum.

The Liebermann-Burchard test for cholesterol and its esters shows a positive reaction especially in the old sebum. In this respect, the distribution of sterols in the sebum follows that of the triglycerides as described above.

The birefringent crystals are more abundant in the old sebum than in the newly-formed sebum. Seldom do crystals appear as spherocrystals. It is difficult to state which of the lipid substances may be responsible for the anisotropy because any, or all of them, may be in this form (cf. Montagna and Noback, '47). Sections which were immersed in acetone at room temperature for hours still retained some anisotropy in the old sebum.

The distribution of autofluorescent lipids resembles that of birefringence. The highly birefringent old sebum fluoresces bright yellow-orange, whereas the new sebum, which is weakly birefringent, fluoresces with a dull gray-yellow light.

In all of these tests, comparison between the glands of normal adult males with those of castrated males, and castrated females injected with testosterone propionate, reveals that androgen plays an important role in the stimulation and maintenance of the sebaceous glands of hamsters. In the ovariectomized females there were only rare instances in which some glandular acini showed a degree of normal activity. The majority of the acini of these glands were composed of epithelial buds which contained, at most, only traces of the substances named above. After injection of the animals daily with 2 mg of testosterone propionate, the glands became, within 10 days, as large as those of normal males and accumulated all of the products found in the glands of

normal males. In addition, the glands of ovariectomized females which had been injected for 10 days or longer showed a greater degree of activity than those of normal males, as judged by the greater amount of sebum in the acini and in the ducts. Thus the sebaceous glands of the general skin, and to an extraordinary degree the glands of the pigmented spot, have an ability to synthesize and store lipid substances that is highly sensitive to, and dependent upon, androgenic stimulation. This is in keeping with the androgen-sensitivity of sebaceous glands in man, as shown by studies of eunuchs and their responses to androgenic treatment (Hamilton, '41, '42).

The presence of acid phosphatase and the practical absence of alkaline phosphatase in the sebaceous glands of the hamster represents the reverse of the status of these enzymes in the glands of the rat (Montagna and Noback, '47) and of the dog (Montagna and Parks, '48). In the rat and the dog enzyme activity was found to be abundant in the alkaline range and nearly absent in the acid range. Acid phosphatase is especially abundant in the sebum. It appears, furthermore, that acid phosphatase is more abundant in the new sebum than in the old. This fits into the scheme of things described above.

Lipase activity, which is normally scanty in the acinar cells, is abundant in the degenerating cells and in the sebum. This enzyme, apparently, plays an important role in the hydrolysis of lipids in the sebum.

SUMMARY

1. The sebaceous glands in the costovertebral pigmented spot are larger and are more active in adult males than in adult females. In females castrated at an early age, the sebaceous glands are small and inactive, containing negligible amounts of lipids.

2. In normal males the sebaceous glands of the costovertebral spot, when stained with Sudan IV, show 4 well defined zones which, proceeding from the periphery toward

the center, are: (a) a peripheral band of cells which contain practically no sudanophilic particles; (b) a narrow zone containing large highly sudanophilic globules; (c) a weakly sudanophilic ring, and (d) the highly sudanophilic degenerating cells and sebum in the center of the glands. After staining with Nile blue sulfate, many of the large lipid droplets in the sebaceous cells, and in the sebum appeared pink. The sebum is anisotropic, and it fluoresces with a bright yellow light. Some birefringence and yellow fluorescence are also present in the degenerating sebaceous cells, but the rest of the glands show neither birefringence nor conspicuous autofluorescence. Positive black granules are formed in the sebaceous cells when tissues are treated with the Fischler method. The large lipid droplets remain unstained but they are surrounded by a black film. The degenerating sebaceous cells and the new sebum become black. The older sebum near the exit of ducts is colorless. The distribution of acid-hematein-positive substances (phospholipids) in the sebaceous cells resembles that of the mitochondria and Golgi bodies described by other authors. Strong plasmal reaction is found only in the sebum.

3. In castrated females the sebaceous glands of the costovertebral spot contain minimal amounts of demonstrable lipid substances. Only the sebum and a few degenerating sebaceous cells are sudanophilic, stain pink after treatment with Nile blue sulfate, are birefringent, emit a yellow fluorescent light, contain Fischler-positive and acid-hematein-positive substances, and some plasmal.

4. The differences between the glands of normal males and those of castrated females are corrected where the castrated females are injected with androgen. After treatment for 10 days the glands of castrated females resemble those of normal adult males.

5. In normal males, in ovariectomized females and in androgen-treated ovariectomized females the sebum and the degenerating sebaceous cells show abundant lipase and acid

phosphatase activity; alkaline phosphatase activity is found only at the periphery of the sebaceous glands.

6. Cytoplasmic basophilia bears an inverse relation to the accumulation of lipid droplets in the sebaceous cells.

LITERATURE CITED

- ALBERT, S., AND C. P. LEBLOND 1946 The distribution of the Feulgen and 2, 4-dinitrophenylhydrazine reactions in normal, castrated and adrenalectomized and hormonally treated rats. *Endocrin.*, 39: 386-400.
- BAKER, J. R. 1944 The structure and chemical composition of the Golgi element. *Quart. J. Micro. Sci.*, 85: 1-71.
- 1946 The histochemical recognition of lipine. *Quart. J. Micro. Sci.*, 87: 441-470.
- 1947 Further remarks on the histochemical recognition of lipine. *Quart. J. Micro. Sci.*, 88: 463-465.
- BOWEN, R. H. 1926 Studies in the Golgi apparatus in gland-cells. II. Glands producing lipoidal secretions—the so-called skin glands. *Quart. J. Micro. Sci.*, 70: 193-215.
- 1929 The cytology of glandular secretion. *Quart. Rev. Biol.*, 4: 484-519.
- CAIN, A. J. 1947a The use of Nile blue in the examination of lipoids. *Quart. J. Micro. Sci.*, 88: 383-392.
- 1947b An examination of Baker's acid hamatein test for phospholipines. *Quart. J. Micro. Sci.*, 88: 467-478.
- CASPERSSON, T., H. LANDSTRÖM-HYDEN, AND L. AQUILONIUS 1941 Cytoplasmannukleotide in Eiweissproduzierenden Drüsenzellen. *Chromosoma*, 2: 111-131.
- FISCHLER, F. 1904 Über die Utersecheidung von Neutralfetten, Fettsäuren und Seifen in Gewebe. *Zentralbl. f. allg. Path. u. path. Anat.*, 15: 913-917.
- GOMORI, G. 1941a The distribution of phosphatase in normal organs and tissues. *J. Cell. and Comp. Physiol.*, 17: 71-83.
- 1941b Distribution of acid phosphatase in the tissues under normal and under pathologic conditions. *Arch. Path.*, 32: 189-199.
- 1942 Histochemical reactions for lipid aldehydes and ketones. *Proc. Soc. Exp. Biol. and Med.*, 51: 133-134.
- 1946 Distribution of lipase in tissues under normal and under pathologic conditions. *Arch. Path.*, 41: 121-129.
- HAMILTON, J. B. 1941 Male hormone substance: a prime factor in aene. *J. Clin. Endocrinol.*, 1: 570-592.
- 1942 Male hormone stimulation is prerequisite and an incitant in common baldness. *Am. J. Anat.*, 71: 451-480.

- HAMILTON, J. B., AND W. MONTAGNA 1949 Secrogogic effects of androgens upon sebaceous glands as studied in hamsters, with supplementary observations as to effects upon pigment, connective tissues, vascularity and muscle of the dermis. I. Morphologic changes. *Am. J. Anat.* (In press.)
- KUPPERMAN, H. S. 1944 Hormone control of a dimorphic pigmentation area in the golden hamster (*Cricetus auratus*). *Anat. Rec.*, 88: 442. (Abstract.)
- LILLIE, R. D. 1948 Histopathologic technic. Blakiston, Philadelphia.
- LISON, L. 1936 Histochemie animale. Gauthier-Villars, Paris.
- LUDFORD, R. J. 1925 The cytology of tar tumors. *Proc. Roy. Soc. London*, 98B: 557-577.
- MONTAGNA, W., AND C. R. NOBACK 1946 The histochemistry of the preputial gland of the rat. *Anat. Rec.*, 96: 111-128.
- 1947 Histochemical observations on the sebaceous glands of the rat. *Am. J. Anat.*, 81: 39-62.
- MONTAGNA, W., AND H. F. PARKS 1948 A histochemical study of the glands of the anal sac of the dog. *Anat. Rec.*, 100: 297-318.
- NICOLAS, J., C. REGAUD, AND M. FAVRE 1914 Sur les mitochondries des glandes sébacées de l'homme et sur la signification général de ces organites du protoplasma. XVIIth Int. Congr. Med., Sec. 13, Derm. and Syph., pp. 101-104.
- SMITH, J. L. 1908 On the simultaneous staining of neutral fat and fatty acid by oxazine dyes. *J. Path. Bact.*, 12: 1-4.
- 1911 The staining of fat by Nile-blue sulphate. *J. Path. Bact.*, 15: 53-55.
- WOLF, A., E. KABAT AND W. NEEMAN 1943 Histochemical studies on tissue enzymes. III. A study of the distribution of acid phosphatases with special reference to the nervous system. *Am. J. Path.*, 191: 423-439.

PLATES

PLATE 1

EXPLANATION OF FIGURES

1 Sebaceous gland from the costovertebral spot of an adult male hamster, frozen section stained with oil-blue N. Note the concentric stratification labelled A, B, C, and D. 112.5 \times .

2 Same as figure 1, except stained with Sudan IV. Stratification of glands is not as clear. 91 \times .

3 The costovertebral spot of an adult female castrated at an early age, frozen section stained with Sudan IV. Arrows indicate the small sudanophilic areas. The bulk of the glandular substance contains no demonstrable lipids. 91 \times .

4 Sebaceous glands in the spot of a castrated female which had received androgenic injections for 5 days. Note the increase in sudanophilia over figure 3. 91 \times .

5 Glands of castrated female receiving androgenic injections for 10 days. These glands are comparable with those of normal males in size and in the distribution of sudanophilic particles. Observe the stratification of lipid globules and compare with figures 1 and 2. 91 \times .

6 Smith-Dietrich test for phospholipids. The black-staining elements are considered positive. Observe positive reaction principally in the sebium and the degenerating sebaceous cells. 112.5 \times .

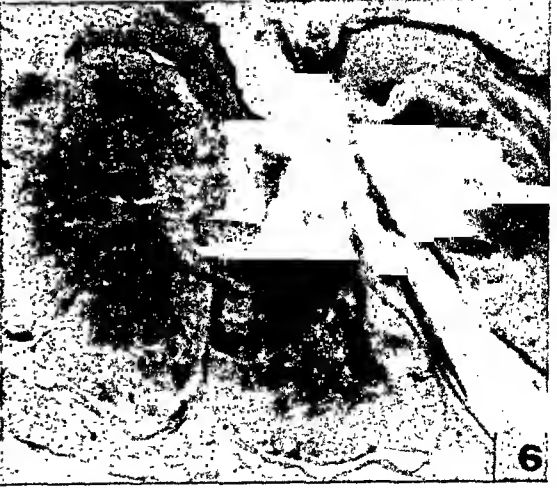
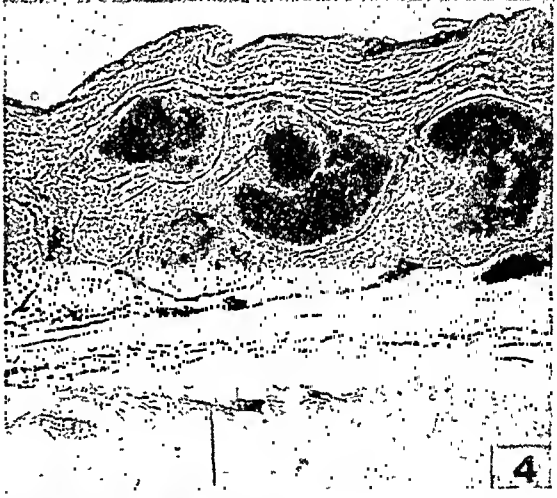
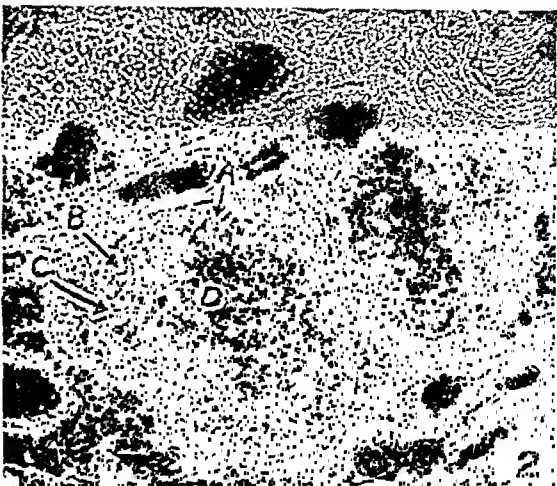


PLATE 2

EXPLANATION OF FIGURES

7 Detail of gland of normal male treated with osmium tetroxide-chromic acid and post-osmicated for 5 days. 1, indicates osmiophilic particles around the nucleus; 2, shows large osmiophilic globules which completely surround nucleus; 3, osmiophilic lipid globules are slightly osmiophobic in the center; 4, lipid globules osmiophobic but they are surrounded by an osmiophilic ring. 1012.5 X.

8 Birefringence in sebaceous glands of normal male hamster. Anisotropy mostly in the degenerating sebaceous cells and sebum. 91 X.

9 Baker's acid-hematein test for phospholipids. Observe positive black particles in cytoplasm and surrounding lipid globules. 1620 X.

10 DaFano method for Golgi substance. Two mature sebaceous cells. Compare with figure 9. 1620 X.

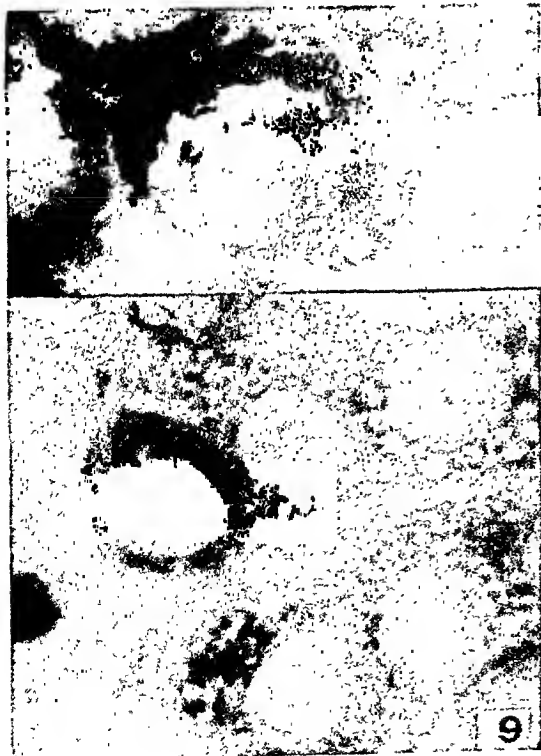
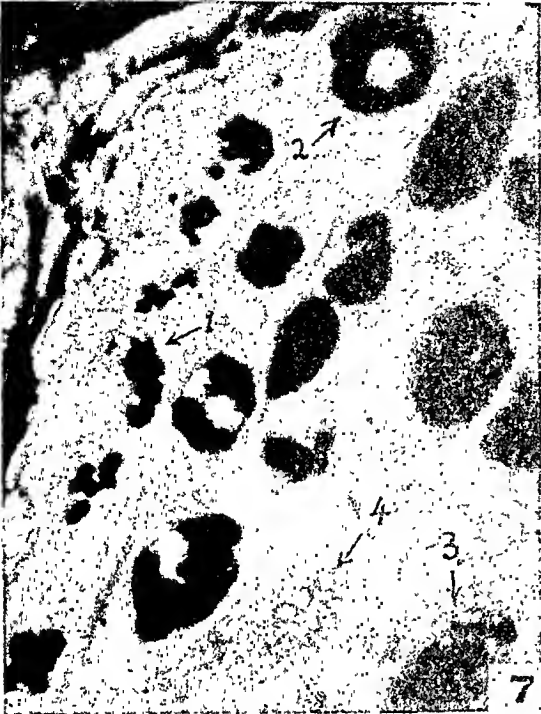


PLATE 3

EXPLANATION OF FIGURES

11 Lipase activity in sebaceous glands of the spot of an adult castrated female. Counterstained with paracarmin. 91 X.

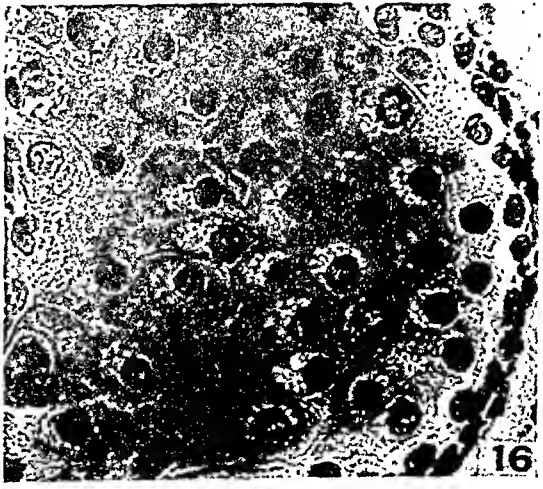
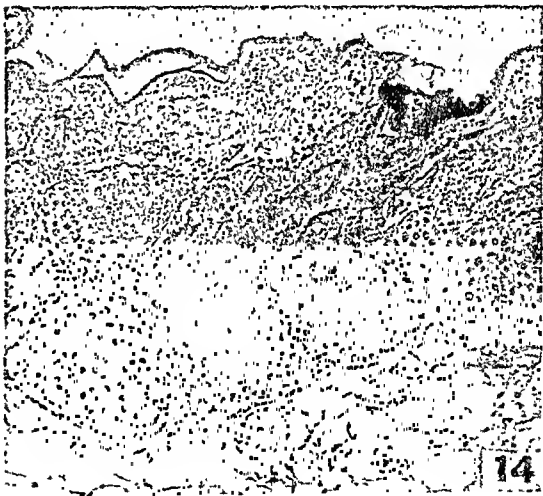
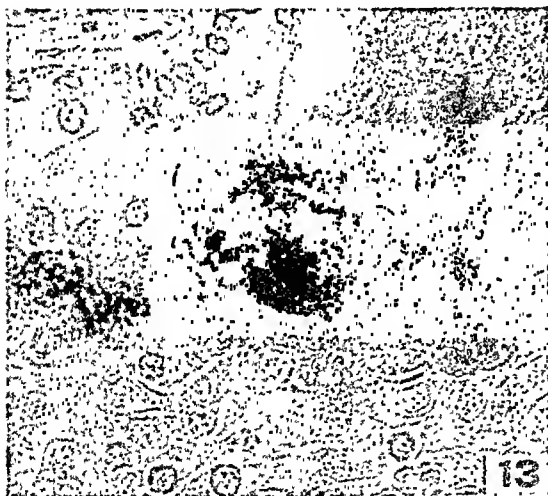
12 Lipase activity in sebaceous glands of an adult male. Counterstained with paracarmin. 91 X.

13 Enlarged detail of figure 12. 455 X.

14 Acid phosphatase activity in sebaceous glands of a castrated female treated 5 days with testosterone propionate. 91 X.

15 Acid phosphatase activity in the glands of normal male. 91 X.

16 Enlarged detail of figure 15. 819 X.



MITOTIC ACTIVITY IN THE LIVER OF THE MOUSE DURING INANITION FOLLOWED BY REFEEDING WITH DIFFERENT LEVELS OF PROTEIN^{1,2}

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ELEVEN FIGURES

INTRODUCTION

The mammalian liver can be stimulated to mitotic activity by a variety of experimental procedures. This, in the full-grown animal, represents an initiation of growth by cell multiplication in an organ which is normally quiescent. That a relation exists between liver growth by mitosis and changes in the alimentation of the animal was reported as early as 1889 by Morpurgo. Liver weight rapidly decreases during fasting (Jackson, '25) and it varies with the level of dietary protein intake (Luck, '36). On the basis of tissue analyses these changes have been ascribed to the loss of or increase in the liver protein (Addis, Poo and Lew, '36a, b, c; Kosterlitz and Cramb, '43). Harrison and Long ('45) and Kosterlitz ('47) have shown that in animals maintained on protein-free or low protein diets a loss of liver cell cytoplasm occurs which may be comparable to the loss of cell proteins during fasting.

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³Horton-Hallowell Fellow of Wellesley College, 1947-48.

⁴National Cancer Institute Postdoctorate Research Fellow, 1948-49.

The present investigation is a cytological study of the liver under these conditions. It was undertaken in order to determine whether the increase in liver size or weight which follows increased protein alimentation involves an increase in the number of hepatic cells through mitotic division or if it represents simply an increase in the size of the original cells through storage of materials.

Two types of experiments have been conducted. Morpurgo (1889) reported that during starvation the mitotic figures in the livers of young rabbits become reduced in number or are lacking entirely but reappear upon subsequent alimentation. This work has been repeated in the mouse, using both young mice in which mitotic figures are still present in the liver and adult mice in which mitotic activity in the liver has normally ceased. After different periods of total inanition the mice were fed diets containing various levels of protein. In the second type of experiment young mice were maintained for varying periods of time on low protein diets and then, after this period of partial inanition, on diets containing higher levels of protein.

In an attempt to find relationships between processes associated with alimentation and mitotic activity, studies were made in some of these experiments of the basophilic component of the cytoplasm of the hepatic cells which represents ribonucleic acid, because ribonucleoprotein is believed to be associated with protein synthesis (Brachet, '42). Cytological observations were also made of glycogen and fat storage in the hepatic cells.

I wish to thank Prof. J. Walter Wilson for his constant encouragement and many suggestions during the course of the investigation.

MATERIAL AND METHODS

The animals used in these experiments were albino mice which were raised in this laboratory. They were maintained at all times at a temperature of 75-78°F. in open screen

cages with raised floors. Except when given special diets the mice were fed Purina laboratory chow. In all of the experiments one or two families of mice of the same age that had been kept together were used. Mice of both sexes were used indiscriminately.

In the fasting experiments one or more mice were used as controls and the rest were starved for a specified period of time. During this period all food was withheld, but water was available at all times. At the end of the fasting period one or more animals were sacrificed. The rest were refed, *ad libitum*, and sacrificed at daily intervals during the re-feeding period. In the experiments in which the mice were

TABLE 1
Composition of semisynthetic diets

| | Basal 1A | High protein 1B | Low protein 1C | Basal 2A | High protein 2B | Low protein 2C |
|---|-------------|-----------------------|----------------------|-------------|-----------------------|----------------------|
| Casein ¹ | 20 | 60 | 3.5 | 30 | 59 | 2 |
| Corn starch (Argo) | 42 | 2 | 58.5 | 29 | | 57 |
| Powdered brewers yeast ¹ | 8 | 8 | 8 | 8 | 8 | 8 |
| Salt mixture ¹ (USP XII, #2) | 4 | 4 | 4 | 7 | 7 | 7 |
| Crisco | 24 | 24 | 24 | 20 | 20 | 20 |
| Corn oil (Mazola) | | | | 4 | 4 | 4 |
| Cod liver oil (Squibb's) | 2 | 2 | 2 | 2 | 2 | 2 |

¹ Obtained from General Biochemicals, Inc.

maintained on low protein diets and subsequently given diets containing higher levels of protein *ad libitum*, animals were sacrificed at 6-hour intervals during the first day on increased protein and at daily intervals thereafter.

Semisynthetic diets were employed which were isocaloric but which varied in protein content. The composition of these diets is presented in table 1. In early experiments the basal diet (1A) was based on that of Harrison and Long ('45). High and low protein modifications (1B and 1C) were made by substitutions of protein for equivalent amounts of carbohydrate. The basal diet was changed in all later experiments (2A) to increase the protein and salt content

(Beard, '26) and to insure the presence of unsaturated fatty acids (Burr and Burr, '30). The high and low protein diets (2B and 2C) were varied accordingly. Yeast protein, which amounts to 3.9% of the total diet (Harrison and Long, '45) was present in all of the diets.

When sacrificed the animals were stunned by a blow on the head, decapitated and bled thoroughly. Small blocks of tissue from the large left lateral lobe of the liver were immersed in the fixing fluids. Except where mentioned the animals were always sacrificed at the same time of day to avoid any diurnal variations.

As a routine all livers were fixed in Bouin's fluid and stained with Delafield's hematoxylin and eosin. In some experiments the following techniques were also employed: (1) For the demonstration of glycogen the liver was fixed in picro-alcohol-formalin and stained by the Bauer-Feulgen technic. (2) For the demonstration of neutral fat the tissue was fixed in Baker's ('44) formol-calcium fixative, imbedded in gelatin, sectioned at 10μ on the freezing microtome and stained with Sudan black. (3) For demonstrating the basophilic fibrils in the cytoplasm the liver was fixed in Zenker's fluid, sectioned at 5μ and stained in a M/16,000 solution of methylene blue buffered at pH 8.1 with phosphate buffers (ionic strength of 0.01). The sections were stained in 1000 ml of this solution for 24 hours at room temperature with constant stirring. Control sections were treated with a 0.1% solution of ribonuclease in an acetate veronal buffer (pH 6.78) before staining.

The mitotic activity of the livers was determined by the method of Wilson and Leduc ('47). Bouin preparations were used for this purpose. All the mitotic figures in a single 5μ section of each liver were counted under an oil immersion lens and the mitotic activity was computed by reducing the number of mitotic figures to a standard area and nuclear density, according to the following formula:

$$\text{mitotic activity} = \frac{\text{number of mitotically active nuclei}}{\text{area of section} \times \text{nuclear density of section}}$$

This is an arbitrary number, but it is adequate as a comparative measure. The above procedure would not warrant the calculation of the mitotic index, that is, the number of mitotically active cells per thousand. The computed mitotic activity is, however, approximately one-half the mitotic index.

The nuclear density was determined by counting in three fields the number of cells outlined by a Howard disc at a magnification of $675\times$. The reciprocal of the nuclear density (Deane, '44) was used as an approximate measure of cell size.

EXPERIMENTS AND OBSERVATIONS

Fasting in young mice. To study the changes in mitotic activity in the liver of the young mouse during fasting, two

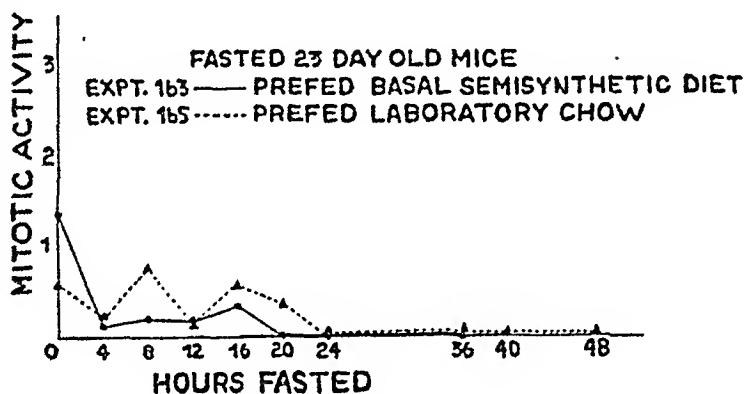


Fig. 1 Mitotic activity in the livers of fasting, 23-day-old mice.

litters of 23-day-old mice were used, one prefed with a basal semisynthetic diet for 4 days and one prefed entirely with laboratory chow. Control animals were sacrificed at 9:00 P.M. and all food was withheld from the remaining animals thereafter. The fasting mice were sacrificed at 4-hour intervals during the fasting period. The changes in mitotic activity in the livers of these animals are presented graphically in figure 1. Each point on the curves represents the mitotic activity observed in one animal. Mitotic figures in the livers of the young mice persisted through 20 and 24 hours after food was withdrawn, but no mitotic activity was subsequently observed. The nuclear density in these livers increased gradu-

ally, which indicated a decrease in cell size. Mice of a third litter of the same age, but with food available at all times, were sacrificed at the same intervals in order to make certain that the changes observed were due to fasting and not to normal diurnal variations. Mitotic activity in the livers of these animals was essentially like that in the livers of the fasting mice during the first 16 hours, but mitotic figures never disappeared entirely as they did in fasting mice.

Fasting and refeeding. To determine the effect on mitotic activity of refeeding after a fast, 4 groups of experiments were conducted in which mice of different ages were fasted and subsequently refed with different diets. In the first group mice of different ages were refed with laboratory chow (which contained 26% protein). Semisynthetic diets, varying in protein content, were fed in the remaining three groups of experiments to weanling mice, to young adult mice and to somewhat older adult mice.

Refeeding with laboratory chow. Three experiments were conducted in which the animals were fasted and subsequently refed with laboratory chow. Nine 23-day-old mice were fasted for 24 hours during which time they lost an average of 14% of their original body weight. Mitotic activity was present in the livers of the control, non-fasted mice, but there was none in the liver of the animal which was sacrificed at the end of the fasting period or after one and two days of refeeding (fig. 2, exp. 75). Mitotic activity reappeared on the third day of the refeeding period, rose to a peak on the 5th and 6th days and subsided to the control level on the 7th and 8th days of refeeding. Similar results were obtained with two groups of older mice, 10 in each group, 44 and 98 days of age (fig. 2, exp. 113 and 114), which were fasted for 48 hours and subsequently refed the laboratory chow. During the fasting period they lost an average of 24% and 17% respectively, of their original body weight. There were no mitotic figures in the livers of the controls, the fasted animals or those which had been refed for one to three days. Mitotic activity appeared on the 4th day of refeeding and

was greatest on the 5th day. The size of the hepatic cells had decreased by approximately 50% in the young mice and by approximately 30% in the older mice at the end of the fasting period. In the mice which were refed for one day cell size returned to and even surpassed slightly that of the control animals. There were no marked variations in cell size during the remainder of the refeeding period.

Refeeding with semisynthetic diets. To determine the effect of refeeding starved animals with protein at different levels, semisynthetic diets were used which were isocaloric but which

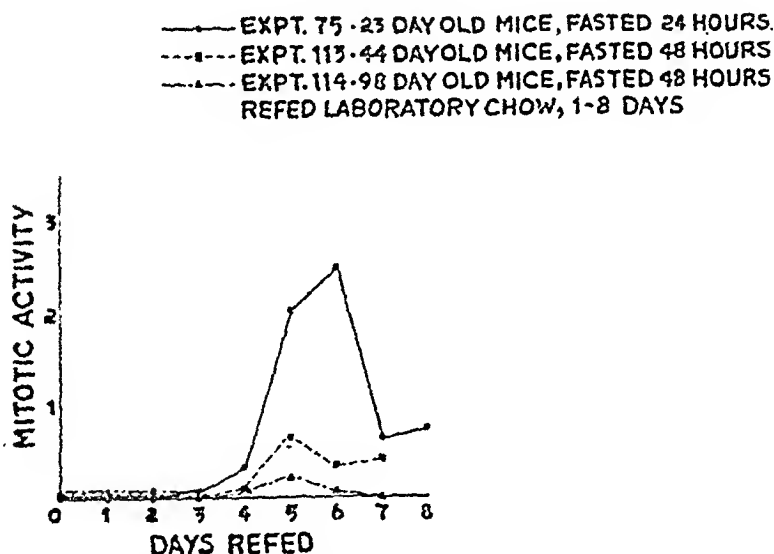


Fig. 2 Mitotic activity in the livers of mice which were refed with laboratory chow following a period of fasting.

varied in protein content. In all of these experiments the animals were prefed for a period of 5 to 6 days on the basal semisynthetic diet (2A) containing 30% casein plus 3.9% yeast protein in order to accustom them to this type of alimentation. The experimental animals were then fasted for a definite period of time (young mice, two days; adult mice, three days and 5 days) and subsequently refed with one of the following diets: (1) the basal ration (33.9% protein), (2) the low protein diet (2C) containing 2% casein and 3.9% yeast protein, (3) the high protein diet (2B) containing 59%

casein and 3.9% yeast protein. One refed mouse on each diet was sacrificed every 24 hours during a refeeding period of 7 or 8 days. Control, non-fasted mice, maintained on the basal ration, were killed at intervals during the experiments.

In three experiments young, weanling mice, 20 to 22 days of age, were used. Two litters, or a total of 12 to 15 animals, were used in each experiment. At the end of the two-day fasting period the three groups of mice which subsequently

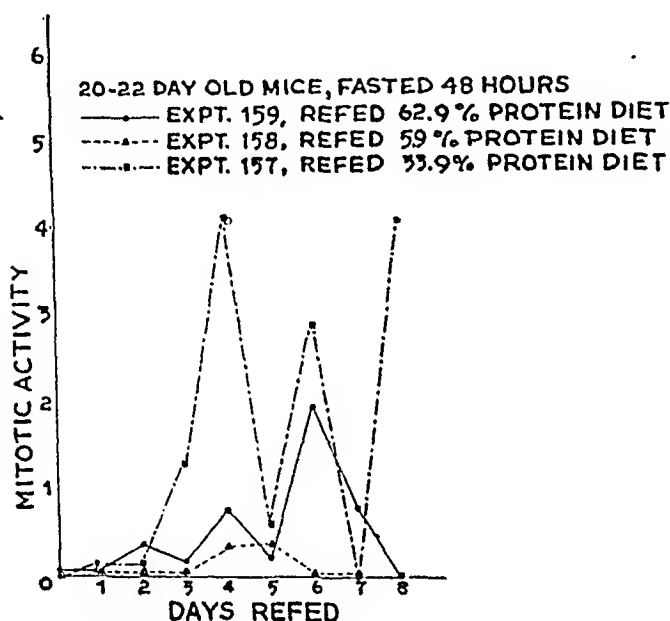


Fig. 3 Mitotic activity in the livers of weanling mice which were refed with basal (2A), high (2B) and low (2C) protein diets following a 48-hour fast.

were refed the basal, low and high protein diets had lost an average of 21%, 25% and 27% respectively, of their original body weight. The variations in mitotic activity in the livers of these animals are presented in figure 3. Mitotic figures were lacking entirely in the livers of the mice which were sacrificed after 24 and 48 hours without food in all three of the experiments, although a small amount of mitotic activity was present in all of the non-fasted control animals. In the mice which were fasted and subsequently refed the basal

semisynthetic diet (exp. 157) mitotic activity reappeared after the first and second days of refeeding, rose to a peak on the 4th day, and subsided a little but remained high on the 6th and 8th days. In the animals which were refed the low protein diet (exp. 158) a very small amount of mitotic activity reappeared on the 4th and 5th days only. In those refed with the high protein diet (exp. 159) mitotic figures reappeared in the liver on the second day and persisted through the rest of the refeeding period. Mitotic activity was at no time very high but it reached a peak on the 6th day of refeeding. The mitotic activity at the peak

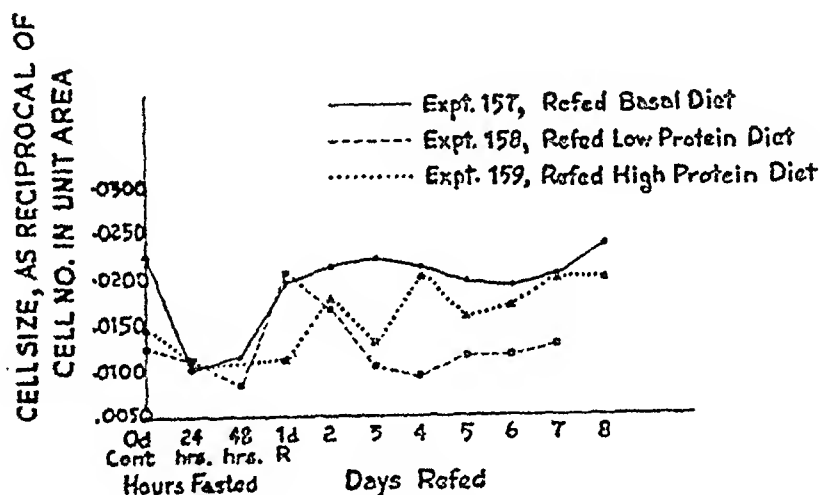


Fig. 4 Changes in cell size in the livers of weanling mice which were fasted for 48 hours and subsequently refed with basal, high and low protein diets.

was 4.1 in the mice which were refed the basal diet, 0.39 in those refed the low protein diet and 1.98 in those refed the high protein diet.

The hepatic cells decreased rapidly in size during fasting (fig. 4). In the animals which were refed the basal diet and in those refed the high protein diet the cells increased after one day of refeeding to approximately their original size and in general maintained this size during the remainder of the refeeding period. There was, however, considerable variation in hepatic cell size in those receiving the high protein ration. Cell size rose rapidly during the first day

of refeeding in the livers of the mice refed the low protein diet, but dropped nearly to fasting level by the third day and remained low through the rest of the refeeding period.

Three experiments were conducted in which young adult mice, 72 to 88 days of age, were fasted for three days and subsequently refed the semisynthetic diets containing different levels of protein (fig. 5). In experiment 154, 11 mice, 80 and 82 days of age, were used. The fasted mice were refed the basal diet. Fourteen mice, 74 to 88 days of age, were used in experiment 155 in which the fasted animals were

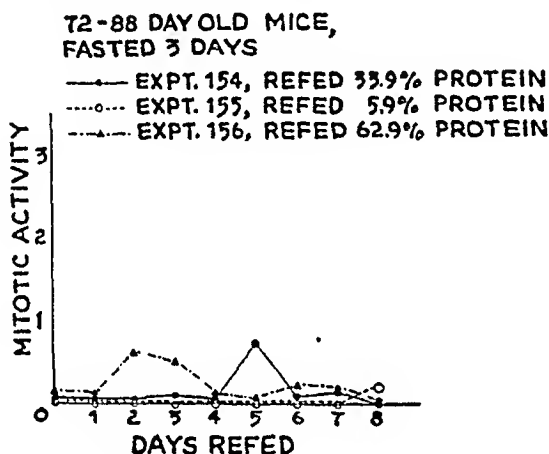


Fig. 5 Mitotic activity in the livers of young adult mice which were refed with basal (2A), high (2B) and low (2C) protein diets following a 3-day fast.

refed the low protein diet. In experiment 156, in which 12 mice, 72 and 74 days of age, were used, the fasted animals were refed the high protein diet. Two of the mice in each experiment served as non-fasted controls. At the end of the three-day fasting period the average body weight loss in each experiment was 25% of the original body weight. In these experiments with adult mice no mitotic figures were found in the livers of the non-fasted control mice or in the fasted mice. In the fasted mice which were refed the basal semisynthetic diet a small amount of mitotic activity in the liver was found on the third to the 7th days of refeeding with a peak (0.72) on the 5th day (fig. 5, exp. 154). In the

animals which were refed the low protein diet there was no significant mitotic activity in the liver. A very small amount of activity (0.05) occurred on the 8th day of the refeeding period, the last day of the experiment (fig. 5). Mitotic activity appeared on the second day of refeeding with the high protein diet (fig. 5, exp. 156) and was highest on that day (0.6). It gradually subsided during the remainder of the refeeding period.

Refeeding with low and with high protein diets was repeated using older mice which were fasted for a longer period of time. The animals were 113 to 125 days of age and

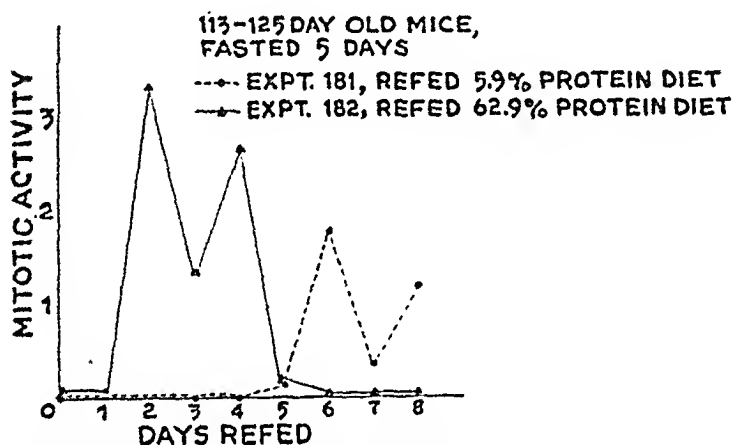


Fig. 6 Mitotic activity in the livers of adult mice which were refed with a high (2B) and a low (2C) protein diet following a 5-day fast.

they were fasted for 5 days. A total of 24 mice was used: three non-fasted controls, 5 sacrificed at daily intervals during the fasting period (two succumbed), 6 refed the low protein diet and 8 refed the high protein diet. The animals refed with the low protein and the high protein diets had lost 27% and 29% of their original body weight, respectively, at the end of the 5-day fasting period. No mitotic figures were found in the livers of the non-fasted controls or of the fasted animals. The changes in mitotic activity in the livers of the refed mice are presented in figure 6. Two mitotic figures were found in a section of the liver after three days of refeeding on the low protein diet (exp. 181) and one was

found after 4 days. These figures fall within the limits of the expected rare occurrence of mitosis in adult mouse livers. A small amount of mitotic activity appeared on the 5th day of low protein alimentation and was greatest on the 6th and 8th days (1.8 and 1.2, respectively). In experiment 182 in which the fasted mice were refed the high protein diet mitotic activity first appeared on the second day of refeeding and was highest on that day (3.37), although it continued to be high through the 4th day. It then quickly subsided.

In all of the adult mice the hepatic cells decreased markedly in size during fasting. The changes in cell size after the mice were refed with the diets containing different protein levels were similar to those observed in the younger mice (fig. 4). For example, in the mice refed the basal diet the average liver cell size rose to slightly above the original value after the first day of refeeding and then leveled off. In those refed the high protein diet the increase in cell size was more gradual, while in those refed the low protein diet the cells increased to nearly their original size on the first day of refeeding, but decreased gradually after the third to the 5th days.

Glycogen, fat and cytoplasmic basophilia. Observations of glycogen, fat and nucleoprotein fibrils or masses were made in all the preceding experiments in which semisynthetic diets were fed to the fasted mice. Since the changes with each diet followed essentially the same pattern in the mice of different ages, these observations are described together.

Glycogen, in the form of either discrete, fine granules or larger aggregates or plaques, was present in small to moderate amounts in the livers of nearly all non-fasted, control mice. In the fasting mice it was present in small amounts for as long as three or 4 days, but there was none after 5 days. During the fasting period the hepatic glycogen was located chiefly in the peripheral zone of the lobule, in the form of a few isolated, very large granules. When the fasted mice were refed the basal diet there was a massive deposition of glycogen in nearly all cells of the lobule. This accounts

for the very rapid increase in size of the hepatic cells during the first day of refeeding. After the second or third day the hepatic glycogen gradually decreased in amount and became somewhat more concentrated in the central cells. Large amounts of glycogen were similarly deposited in all the liver cells after one day of refeeding with the low protein diet and this condition persisted throughout the refeeding period of 8 days. Very little glycogen was deposited in the livers of the animals refed on the high protein diet. The small amounts that were present were concentrated around the central vein of the lobule.

Neutral fat was present in the livers of the non-fasted, control mice in very small amounts and was localized chiefly in the centrolobular cells. There was considerable individual variation in the fat content of the livers of the fasting mice, but, in general, in both young and adult mice marked fatty infiltration of the liver had occurred after 24 hours of fasting. The fat was present in very numerous discrete droplets evenly distributed throughout the lobule. Usually only small amounts of neutral fat were found after two days of fasting and there was extremely little or none in the adult mice fasted for three or 4 days. In the livers of all the mice which were fasted and subsequently refed, very little fat was found during the first two days of refeeding. On the third and 4th days fat was abundant and filled the hepatic cells with large, irregular, coalesced drops of sudanophilic material. This fat decreased in amount on the subsequent days of refeeding, except in those animals receiving the low protein diet where it remained in relatively large amounts, and became localized in the portal and midzonal cells of the lobule.

Basophilic material is abundant in the liver cell cytoplasm of the well-fed mouse. In the central cells of the lobule this basophilic material appears as large, irregular clumps or masses and in the portal cells, as fine fibrils (after acid fixation). These masses and fibrils fail to stain with basic dyes after treatment of the sections with ribonuclease, which indicates that they contain ribonucleic acid and probably con-

sist of ribonucleoprotein. In the livers of the fasting mice there was a reduction in the amount of this basophilic material and a progressive series of changes in its distribution. During the first 24 hours of fasting there was an initial dispersal of the basophilic masses of the central cells into their constituent fibrils and a gradual diminution in the amount of basophilic material in all cells. After a more prolonged fast fine basophilic fibrils persisted in the peripheral cells of the lobule, but the central cells seemed to contain only a faintly basophilic, granular ground substance. When the mice were refed, aggregates or masses of the basophilic fibrils reappeared in the central cells. The changes which occurred were just the reverse of those which took place during fasting. Basophilic masses reappeared in a narrow zone around the central vein in the adult mice refed, after fasting, with the basal diet for 24 hours and in the young mice after 48 hours. These masses gradually became more abundant and filled a broader zone around the central veins, as was found in the well-fed animals, on the 4th and 5th days respectively in the young and adult mice. This corresponds to the time of appearance of the peak of mitotic activity in the same livers. In both the young and adult mice refed with the low protein diet a few small basophilic bodies were present after 24 hours of refeeding and large masses appeared after the second day. The basophilia of these cells subsequently decreased, however, and only fibrils and a few small aggregates of fibrils were present in the central cells from the 5th to the 8th days. Refeeding a high protein diet after a period of fasting stimulated a very rapid increase in cytoplasmic basophilia in the liver. Either abundant large fibers or large solid masses were found in the central cells after 24 hours. On the second and third days of refeeding, however, there were only very fine fibrils and a lightly stained, basophilic ground substance. This represented a diminution of the basophilic substance throughout the lobule and not simply a change in its distribution. Small basophilic masses reappeared on the 4th day of refeeding and became large

and abundant after the 5th day in the young mice and the 5th and 6th days in the adult mice.

Increased dietary protein level. To study the effect on liver mitosis of an increase from low to basal or high levels of dietary protein 10 experiments were conducted. Ten to 12 mice from two litters were used in each experiment. In all except one of the experiments mitotic activity in the livers of the mice killed at the end of the period of maintenance on low protein diets was either very low or absent.

Two groups of mice (exp. 87 and 88) which were 35 days of age at the beginning of the experiment received a low protein diet (1C) containing 7.4% protein (3.5% casein and

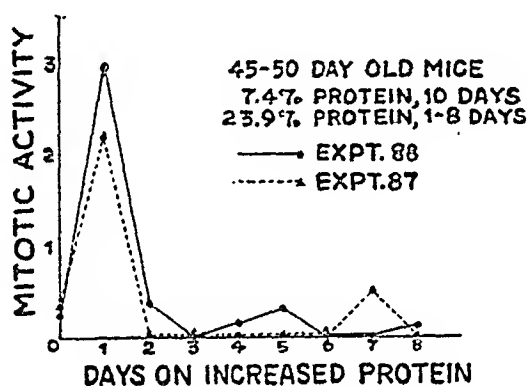


Fig. 7 Mitotic activity in the livers of young mice which were fed a basal diet (1A) following a 10-day period of maintenance on a low protein diet (1C).

3.9% yeast protein) for 10 days. A basal diet (1A) containing 23.9% protein was then substituted for the low protein diet and the mice were killed at daily intervals during the increased protein alimentation. The changes in mitotic activity in the liver are shown in figure 7. Increased activity occurred on the first day of increased protein intake. The mitotic activity increased from less than 0.5 to 2.2 and 2.98 in experiments 87 and 88, respectively, and returned to the original level on the second day.

One group of 36-day-old mice was given the low protein diet (2C) containing 2% casein and the 3.9% yeast protein for 16 days and then this diet was replaced by the basal semisynthetic diet (2A) containing 30% casein plus the

yeast protein. The changes in mitotic activity in the livers are presented in figure 9 (exp. 188). Mitotic figures appeared after 12 hours and persisted through 4 days of the increased protein regime. Activity was greatest after 24 and 48 hours when it was 1.6 and 2.1, respectively.

Two groups of 35-day-old mice (exp. 106 and 107) were fed the 7.4% low protein diet (1C) for 12 days, followed by

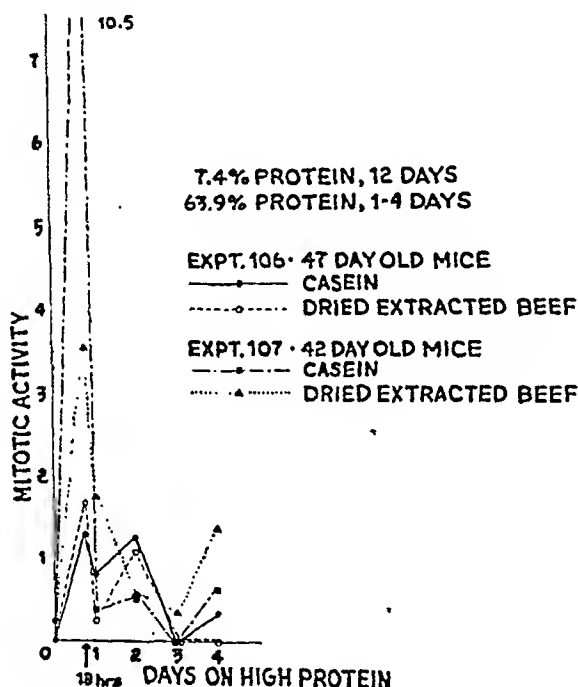


Fig. 8 Mitotic activity in the livers of young mice which were fed high protein diets (1B) following a 12-day period of maintenance on a low protein diet (1C).

a high protein (63.9%) diet (1B). Half of the mice in each experiment received casein and half received dried extracted beef as the major source of protein, but no consistent difference was found in the results with these two types of protein. An increase in the number of mitotic figures in the liver appeared between 6 and 12 hours after the mice were fed the high protein diet and the greatest mitotic activity occurred after 18 hours (fig. 8). Mitotic activity values were

1.3 and 1.7 in experiment 106 and 10.5 and 3.5 in experiment 107. This increased activity persisted through 48 hours of increased protein alimentation in experiment 106. The very large number of mitotic figures counted in one section of the liver of the mouse to which a high level of casein had been available for 18 hours (mitotic activity 10.5, exp. 107, fig. 8) is an example of unexplained explosive activity which is sometimes obtained following various experimental procedures.

Two similar experiments were conducted in which the increased level of protein consisted of casein for one-half of the mice and gelatin, as a protein inadequate for growth, for the other half. The two groups of mice, 36 days of age when given the experimental diet, were maintained on the 7.4% protein diet (1C) for 10 days. Diets containing 63.9% protein (1B) were then substituted for the low protein diet; half of the mice in each experiment received 60% casein and 3.9% yeast protein; the other half, 60% gelatin and the yeast protein. In those receiving the increased level of casein, increased mitotic activity was found in the liver, 1.1 and 1.5 after 24 and 48 hours on one experiment (exp. 105), 1.0 and 1.1 in the other (exp. 109). In those receiving the high level of gelatin, on the other hand, no increase in the number of mitotic figures over that found in the mice on the low casein diet was observed. The average mitotic activity in these animals was less than 0.1.

In the three remaining experiments younger mice, 21 to 25 days of age, in which protein depletion might be expected to take place more rapidly, were used. One group (exp. 187) was maintained on a low protein (5.9%) diet (2C) for 16 days. This diet was then replaced by the basal diet (2A) containing 33.9% protein. Mitotic activity appeared in the liver after 18 hours of increased protein alimentation and remained high throughout the 5-day period in which it was studied (fig. 9). The peak of activity occurred after 24 and 48 hours when it was 5.4 and 6.1 respectively.

In two experiments the young mice were maintained on the low protein (5.9%) diet (2C) for 28 days. Subsequently, one group received the basal diet (2A) containing 33.9% protein (exp. 175) and one group received the high protein (62.9%) diet (2B) (exp. 244). The changes in mitotic activity in the liver during increased protein alimentation are shown

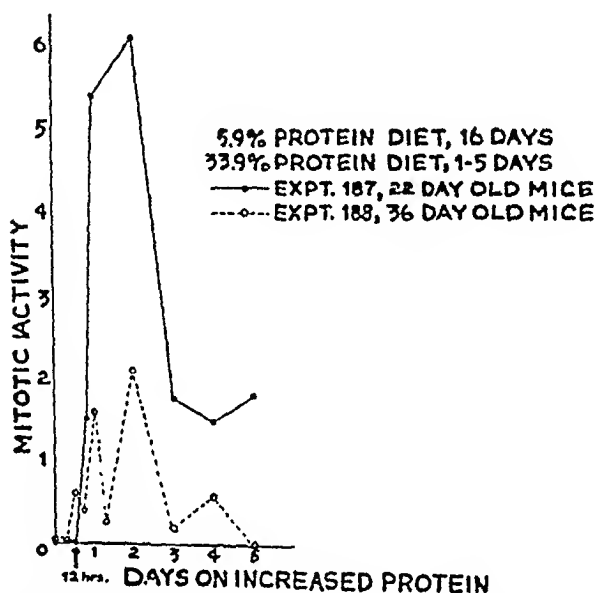


Fig. 9 Mitotic activity in the livers of young mice which were fed a basal diet (2A) following a 16-day period of maintenance on a low protein diet (2C).

in figure 10. In experiment 175 mitotic activity was high on the second, third and 4th days of increased protein with a peak of 8.9 on the third day. In experiment 244 mitotic activity was high in the animal killed at the end of the low protein regime and in those receiving the high protein diet for 24, 36 and 48 hours and 5 days. The mitotic activity on the second day was extremely high (39.5).

The average size of the liver cells in the mice maintained on the low protein diets did not differ essentially from that of animals maintained on basal diets. In those mice which were subsequently fed the basal diet (33.9% protein) cell size diminished slightly during the first 24 hours (fig. 11).

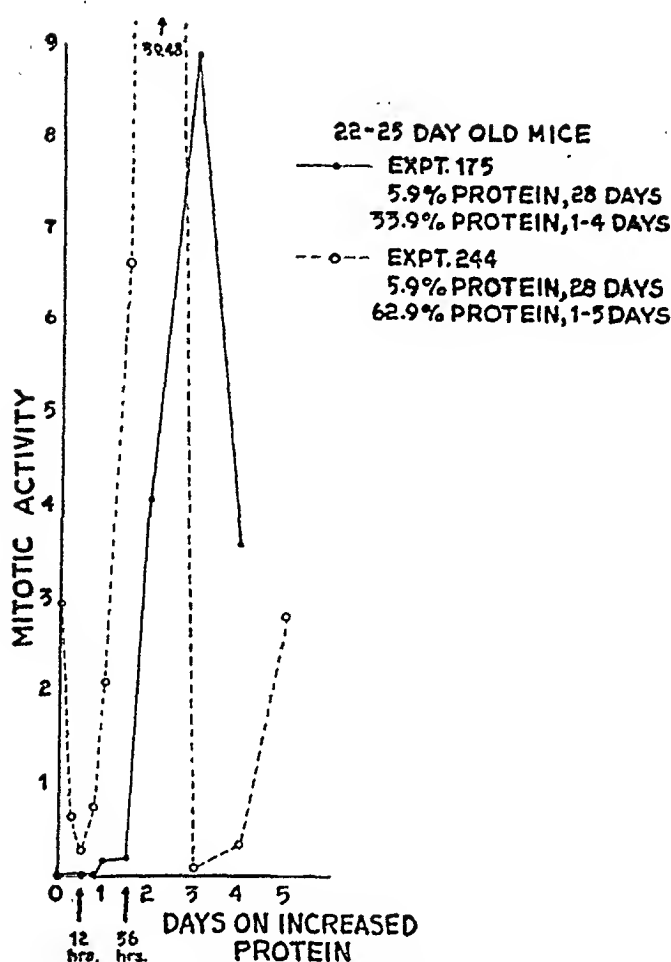


Fig. 10 Mitotic activity in the livers of young mice which were fed a basal diet (2A) and a high protein diet (2B) following a 28-day period of maintenance on a low protein diet (2C).

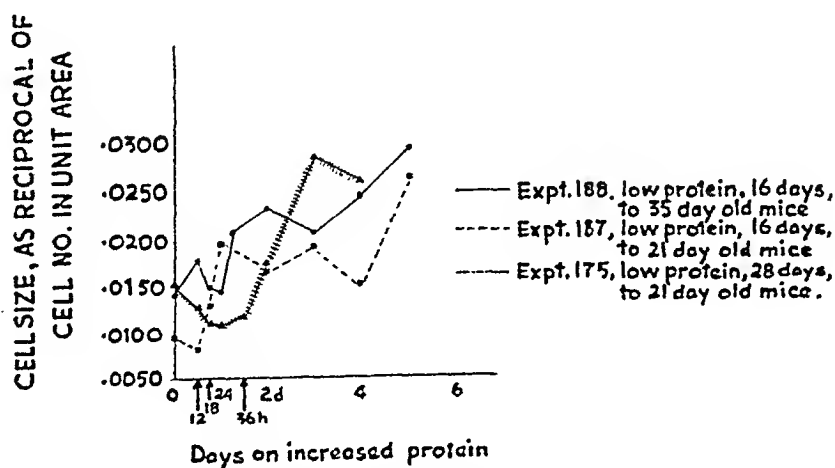


Fig. 11 Changes in cell size in the livers of young mice after increasing dietary protein from a low to a basal level.

During the remaining 4 to 5 days of increased protein feeding the cells gradually increased to nearly twice their original size. Cell size did not increase so markedly in the mice that were fed the high level of protein.

Glycogen, fat and cytoplasmic basophilia. Cytological preparations for the demonstration of glycogen, fat and ribonucleoprotein were made of the livers of the mice in 4 of the experiments involving changes from low to higher levels of dietary protein. These were experiments 188, 187, 175 and 244. In the first two experiments the mice were maintained on the low protein diet for 16 days, in the last two, for 28 days. Those in the first three experiments subsequently received the basal diet and those in the last experiment received the high protein diet.

Glycogen was found in very large amounts in the livers of all animals sacrificed at the end of the period of maintenance on the low protein, high carbohydrate diets. It was present in the form of large plaques in the peripheral and midzonal cells of the lobule and as small granules in the central cells. The glycogen content of the livers dropped during the first 24 to 36 hours of increased protein alimentation. Only fine granules were present which were evenly distributed in the lobule. After two to 5 days moderate to large amounts of glycogen appeared in the form of large granules and plaques in the peripheral and midzonal cells.

The neutral fat content of the livers of mice maintained on the low protein diet was low. It appeared as fine droplets evenly dispersed in the strands of cytoplasm of highly vacuolated cells. Larger drops of sudanophilic material appeared, chiefly in the midzonal cells, during the first 24 hours of higher dietary protein intake. After two to 5 days on the basal diet large amounts of fat were present in nearly all hepatic cells in increasingly larger, coalesced drops. Little fat was present in the livers of the mice that received the high protein diet.

The basophilic masses in the cytoplasm of the central cells of the hepatic lobule were still present after 16 days of feed-

ing on the low protein diet, but after 28 days on this regime they were replaced by fine fibrils, evenly dispersed in the cytoplasm. On the basal diet, after maintenance for 16 days on the low protein diet, there was an initial dispersal of the basophilic masses into very abundant fine fibrils. Deane ('46) has reported such a dispersal of basophilic material in the livers of mice a few hours after feeding. The basophilia in all the cells of the lobule increased during the first day of increased protein alimentation. The fibrils in the central cells reaggregated after 24 hours and large masses were formed by the second and third days respectively in those animals which had previously been maintained on the low protein diet for 16 and 28 days and subsequently given the basal diet. It is at these very times that the peaks of mitotic activity in these livers occurred. Similarly, the restoration of the basophilic ribonucleoprotein masses in the animals given the high protein diet after preliminary maintenance for 28 days on the low protein diet (exp. 244) occurred after 48 hours, at the time of greatest mitotic activity.

DISCUSSION

The experiments reported in this paper show that in young, weanling mice mitotic activity in the liver ceases during starvation and reappears upon subsequent refeeding. These results are in accord with those of Morpurgo (1889) who conducted similar experiments with young rabbits. In adult mice that are refed after fasting mitosis is initiated in the liver. Furthermore, after a short period of maintenance on a low protein diet, a change to a higher level of dietary protein is also followed by a wave of mitotic activity in the liver. This mitotic activity indicates that an increase in the number of cells occurs. In the animals that were fasted a rapid decrease in the size of the liver cells was found. When these animals were refed with basal or high protein diets, the cells returned to their original prefasting size.

That changes in dietary protein induce changes in the protein content of the liver has been demonstrated unequivocally.

cally by Addis, Poo, and Lew ('36), Luck ('36), Kosterlitz ('44a, b; '47), and Harrison and Long ('45). By means of tissue analyses these authors have shown that there is a marked loss of liver protein in fasted animals and in animals maintained on low protein diets, and that it represents a loss of all cytoplasmic proteins rather than the loss of any special storage protein. Kosterlitz ('44b) has called the protein fraction of the liver which is readily lost during early stages of protein inanition "labile" liver cytoplasm. The feeding of a high protein diet results in a protein enrichment in the liver. Luck ('36) has shown that this is the result not only of an increase in the mass of liver tissue but also of an increase in the content of protein per unit weight of tissue. It was suggested by both Luck ('36) and Kosterlitz ('47) that the increase in the mass of liver tissue which follows increased protein alimentation might be the result either of an increase in the size of the liver cells (increase in the amount of cytoplasm) or of an increase in the number of liver cells, or possibly of both. The results of the present investigation show that both do occur.

From the very nature of the experiments here reported variability in the results would be expected. For example, a 48-hour fast in one mouse may be different in its effects from that in another mouse, since even in littermates the individual differences are very great. Ideally, all the changes in mitotic activity should be followed in the liver of a single animal, but it is impossible to use repeated biopsies on the same animal because the partial removal of liver tissue itself induces mitotic activity in the remaining portions of that liver. Therefore, in similar experiments the results are not always exactly the same, particularly in respect to the heights of the peaks of mitotic activity. It is possible that maximum activity is of very short duration so that the animals are not always killed at the very peak of the activity. In spite of these difficulties, however, the general trends of the changes in mitotic activity can be followed.

In the present studies the time of appearance of the peak of mitotic activity was determined by the amount of protein in the diet which was refed. In the adult mice the peak occurred on the second day of refeeding with the high protein diet (62.9%), on the 5th day with the basal diet (33.9% protein), and on the 6th and 8th days with the low protein diet (5.9%). There is an apparent correlation between this appearance of mitosis in the liver and the restoration of liver protein reported by other investigators. Harrison and Long ('45) found that the rate of regeneration of liver proteins after a period of fasting depends in part on the quantity of dietary protein which is refed. In rats previously fasted for two days the liver protein was restored to its original level after 4 days of refeeding with a 40% casein diet but not with a 20% casein diet. Addis, Poo, and Lew ('36c) report the restoration of liver protein after two days of refeeding a 70% casein diet in rats that had fasted for 7 days. Lightbody and Kleinman ('39) found that in rats the liver tissue lost during two days of fasting was not replaced completely by the refeeding of a 6% protein diet for 16 days. Although these studies of protein changes in the liver during fasting and refeeding were carried out on the rat, until evidence to the contrary is presented, it seems reasonable to assume that similar results would be obtained with the mouse. Since these changes in liver protein may be correlated with the observations on mitotic activity that are reported here, it would seem that the production or synthesis of liver protein stimulates, among other things, the formation of new cells through mitotic division.

The relation between the time of appearance of the peak of mitotic activity and the amount of dietary protein which is refed after a period of fasting is not as clear cut in the young, weanling mice as in the adult mice. The peaks of activity in the young mice refed with the low and high protein diets occurred on the 5th and 6th days respectively. Mitosis occurred irregularly in those which were refed with the basal diet and activity was high on the 4th, 6th and 8th days of re-

feeding. The differences between the results in the young and adult animals may be due to the fact that in young mice starvation involves not only reserve or "labile" materials but also the materials needed for actual growth.

The size of the liver cells in both the young and adult mice decreased during fasting and then increased during subsequent refeeding. Kosterlitz and Cramb ('43) found that the decrease in liver size in fasted rats was not caused by a decrease in the number of cells but by a decrease in the volume of each cell, in that the nuclear density per unit volume of tissue increased during fasting. Davidson and Waymouth ('46) reported that while the liver cells in the fasting rat diminished in size, the diameter of the nuclei and the content of desoxyribonucleic acid, a purely nuclear constituent, remained unaltered. In my own experiments the liver cells returned to their original, prefasting size very rapidly in those mice which were refed with the basal diet. This seems to be due to the initial great deposition of glycogen in the liver which distended the cells. This large accumulation of glycogen was present during the first two days that the mice were refed, and then as it gradually subsided there was an increased deposition of neutral fat on the third and 4th days. On the 4th and 5th days, the basophilic material in the cytoplasm which represents ribonucleoprotein was present in the large amounts that are characteristic of the well fed mouse. In the fasted mice that were refed with the high protein diet the increase in cell size was more gradual. This appears to be due to the fact that less glycogen was deposited in the livers of these animals during the early stages of the refeeding period. In the mice refed with the low protein diet, on the other hand, the initial large accumulation of glycogen and the initial increase in cell size occurred, but cell size subsequently diminished gradually. Although a considerable amount of glycogen persisted throughout the refeeding period, the restoration of the cytoplasm itself, and of the constituent ribonucleoproteins in particular, did not occur. From these observations it appears that the ultimate resto-

ration of cell size in the liver depends upon the restoration of the liver protein or cytoplasm.

A change from a low protein diet to one of higher protein content is followed by a rapid increase in mitotic activity in the mouse liver. Under these conditions the time of appearance of the peak of activity appears to be influenced not only by the amount of dietary protein but also by the duration of the period of maintenance on the low protein diet or, in other words, by the duration of the period of protein depletion. In the young mice previously maintained for 28 days on the low protein diet the peaks of mitotic activity occurred 48 hours after the mice received the high protein (62.9%) diet and 72 hours after they received the basal diet (33.9% protein). Therefore, the higher the level of the increased dietary protein, the earlier the peak of activity occurred. The maintenance of animals on a low protein diet, a type of partial inanition, resembles fasting, or total inanition, in that both involve a depletion of liver and body proteins. Harrison and Long ('45) reported that in rats maintained on a low protein diet for one week the liver protein was reduced as much as a 48-hour fast and Kosterlitz ('47) found that a 24-hour fast following maintenance on a low protein diet caused no further decrease in the protein content of the liver. On the basis of my own experiments low protein maintenance also resembles fasting in that with the return of adequate or high levels of dietary protein mitotic activity or growth by cell division occurs in the liver. Furthermore, the longer the mice were maintained on a low protein diet, the longer it took for the peak of mitotic activity to appear after the dietary protein was increased. In the animals that received the basal diet the peaks occurred after 24, 48 and 72 hours in the mice that had previously been maintained on the low protein diet for 10, 16 and 28 days respectively. On the assumption that the longer the maintenance of the mice on a low protein diet, the greater the depletion of body and liver proteins (observations of the basophilic ribonucleoprotein, discussed below, lend support to this assumption) it would seem that the

greater the protein loss and, consequently, the greater the amount which must be restored, the longer it takes for the peak of mitotic activity to appear. In the protein-depleted rat, Harrison and Long ('45) have found that all of the body proteins lost (during fasting) must be restored before complete protein restoration can take place in the liver. The most striking thing in my own experiments is the rapidity with which mitosis appeared. For example, in the mice which were fed the high protein diet after preliminary maintenance for 12 days on the low protein diet mitotic figures appeared in the liver between 6 and 12 hours after the dietary protein was increased and mitotic activity reached a peak after 18 hours. Addis, Poo and Lew ('36c) fed a 74% casein diet to rats that had previously been maintained on a protein-free diet for 10 days and found a large increase in liver protein. This increase was at first very rapid, with signs of an increase being noticeable after 12 hours and definite after 17 hours (Kosterlitz, '47), but it soon became slow. This very rapid increase in the liver proteins under these conditions corresponds to the rapid increase in mitotic activity in the liver of the mouse under similar conditions in the present study.

The size of the liver cells in the mice maintained on low protein diets was essentially the same as that in mice maintained on basal diets. During the first 24 hours of increased protein feeding when the cells diminished in size the glycogen disappeared from the liver. Subsequently, the liver cell content of glycogen, of neutral fat and of ribonucleoprotein gradually and steadily increased. Cell size did not increase so markedly in the livers of the mice that were fed the high protein diet. Considerably less glycogen and neutral fat was found accumulated in these liver cells. As in the animals that were fasted and subsequently refed, the changes in size of the liver cells was due to variations in their cytoplasmic constituents.

A possible explanation of increased liver size following high protein alimentation is that the liver is overworked (Luck, '37) and more cells are needed to perform the func-

tion of deamination of amino acids. This idea is based on an old concept of hyperplasia through hyperfunction which has been applied to a variety of conditions (e.g. Pfuhl, '38; Sternheimer, '39). To test this mice were maintained for short periods of time on a low protein diet and subsequently were fed a diet containing a high level of gelatin, a protein which is inadequate for growth. This change from the low casein diet to a high gelatin diet produced no mitotic activity in the liver. From this observation it might seem that an increased functional load on the deaminating mechanism of the liver is not responsible for the production of new cells in the liver. However, Pearce, Sauberlich and Baumann ('47) have shown that mice fed diets containing proteins which are deficient in essential amino acids excrete very high percentages of all other ingested amino acids in the urine. These apparently are not deaminated in the liver. This would at first seem to indicate that the enzyme systems of the liver really were not overworked. On the other hand, it seems more probable that the functional load was so great that the enzymes present were not able to break down all the amino acids. Furthermore, Miller ('48) found that the enzymes of the liver are lost along with all the cell proteins during fasting in the rat and Kaplansky et al. in 1946 (quoted from Miller, '48) have described the decrease of deaminating, transaminating and glycogen-forming functions in protein-depleted rats. Lightbody and Kleinman ('39) found that when a diet containing gelatin is fed after a two-day fast the concentration of the enzyme, arginase, per unit weight of liver eventually does increase and becomes comparable to that following the ingestion of the same level of an adequate protein, but liver size does not increase. They believe, therefore, that when gelatin-containing diets are fed, the synthesis of new enzymes takes precedence over the restoration of the liver tissue lost during fasting. Harrison and Long ('45) found that after preliminary fasting in rats the refeeding of diets containing gelatin failed to increase the liver nitrogen values above the fasting level. On the basis of the find-

ings of Kosterlitz ('47) and of Harrison and Long ('45) one would expect that under the conditions of my experiments a gradual loss of proteins occurred during the preliminary maintenance of the mice on a low protein diet and it would seem that with this loss of liver proteins there was a decrease in the enzyme systems that normally destroy amino acids. Actually, therefore, during the period when mitotic activity was high following ingestion of casein but absent following the ingestion of gelatin, the deaminating mechanism of the liver cells was overworked, but when the necessary building blocks were present the synthesis of additional enzymes was initiated and the subsequent restoration of liver proteins took place. The increased size of the liver after increased protein alimentation, involving both hypertrophy and hyperplasia may thus be the result, in part at least, of increased function.

The observations of basophilic material in the cytoplasm of the hepatic cells in mice and of its decrease in amount during starvation are in accord with those of Deane ('46) and of Brachet, Jeener, Rosseel and Thonet ('46). Brachet et al. report a corresponding diminution in liver nitrogen and ribonucleic acid during fasting. The basophilic component of this substance is ribonucleic acid, as is shown by the loss of the basophilic staining reaction by digestion of the sections in ribonuclease. Davidson and Waymouth ('46) reported, furthermore, that cytoplasmic masses in the liver which absorb ultraviolet light of the wave length which is characteristically absorbed by nucleic acids disappears during fasting. This is due to a loss of ribonucleic acids, for the desoxyribonucleic acid content of the liver remains unchanged (Davidson and Waymouth, '46). Ribonucleic acid is present in the cytoplasm in ribonucleoprotein (Brachet and Jeener, '44), chiefly in ribonucleoprotein-phospholipid complexes of particulate nature (Claude, '46). This ribonucleoprotein is believed to be associated with the synthesis of cytoplasmic proteins (Brachet, '42; Caspersson, '41). Thus, during fasting when amino acids of alimentary origin are

lacking, cytoplasmic ribonucleoproteins become reduced in amount and protein synthesis declines. The ribonucleoproteins constitute, in part at least, the "labile" liver cytoplasm of Kosterlitz ('44b) but do not represent chemically and physically distinct "storage proteins" in the sense used by Berg ('14).

The basophilic material of the cytoplasm increased in amount when the fasted mice were refed with adequate amounts of protein and when mice previously maintained on low protein diets were fed diets containing higher levels of protein. Similar changes in cytoplasmic ribonucleoprotein bodies in the livers of fasted and refed rats were reported by Lagerstedt ('47) who used the ultraviolet light absorption technic for demonstrating nucleic acids. Lagerstedt found that the absorbing masses which had disappeared during fasting reappeared after 24 hours of feeding, increased in quantity after two days and after 5 days of refeeding did not differ from those in normal animals. Berg ('20) reported that the cytoplasmic masses disappeared during fasting and reappeared when the animals were refed proteins but not when only fat or carbohydrate was refed. The loss of ribonucleoprotein during protein depletion and its restoration with adequate protein alimentation is of particular significance in the light of the discoveries of Jeener and Brachet ('41), Brachet and Jeener ('44), Claude ('46) and others that many of the intracellular enzymes are bound to the ribonucleoprotein-phospholipid particulate material of liver cytoplasm.

The restoration of the basophilic masses in the central cells of the lobule to the appearance of those in normal, well-fed mice occurred at the time of greatest mitotic activity when the fasted mice were refed with the basal diet and when the mice previously maintained on low protein diets were given basal and high protein diets. However, there was no correspondence in time between mitosis and restoration of basophilic masses in the liver cytoplasm in the mice which were fasted and subsequently refed with the low and

high protein diets. Reaggregation of basophilic fibrils into large masses took place on the second day of low protein alimentation in both young and adult mice, but this basophilia subsequently decreased so that only dispersed fibrils or a few small aggregates of fibrils were present from the 5th to the 8th days. Mitotic figures were present on the 4th and 5th days in the young mice and the 6th and 8th days in the adult mice. The presence of mitosis and of the small aggregates of basophilic material, indicates that some protein synthesis was possible even with a low level of dietary protein (5.9%). With high protein realimentation abundant basophilic fibers and large masses reappeared after one day. On the second and third days, however, only very few fine fibrils and a faintly basophilic granular ground substance were present. Large ribonucleoprotein masses reappeared again around the 5th day of refeeding. Mitosis was at a peak on the 6th day in the young mice when cytoplasmic basophilia was high but in the adult mice the peak of mitotic activity occurred on the second day of refeeding at the time when basophilia was greatly reduced. This diminution of cytoplasmic basophilia on the second and third days of refeeding, following the rapid increase on the first day, might be another example of the conditions discussed by Deane ('46). She suggests that the markedly low amount of basophilic material may represent an exhaustion of the enzyme systems as a result of increased protein synthesis. That mitotic activity of the cells should follow or accompany the increase in ribonucleoprotein in the cells is further evidence of the association of mitotic activity in the liver with the synthesis of protein. This was to be expected on the basis of the findings of Brachet ('42) and Davidson and Waymouth ('44) that large amounts of ribonucleic acid are present in rapidly growing tissues where mitotic figures are abundant.

SUMMARY

1. Mitosis, which is normally present in the livers of young, weanling mice, disappears during prolonged fasting

and reappears upon subsequent refeeding. In adult mice that are refed after fasting mitosis is initiated in the liver. The amount of protein in the diet which is refed determines the time of appearance of the peak of mitotic activity.

2. A change from a low protein diet to one containing a higher level of protein is followed by a wave of mitotic activity in the liver. The time of appearance of the peak of mitotic activity appears to be influenced both by the amount of increased dietary protein and by the duration of the preliminary period of maintenance on the low protein diet.

3. In mice maintained for short periods of time on a low diet a subsequent feeding of a diet containing a high level of gelatin produces no mitotic activity in the liver. In spite of an increased functional load on the deaminating mechanism of the liver, unless all the requisites for protein synthesis are present, no increase in the amount of functional liver cytoplasm occurs.

4. The size of the liver cells decreases during fasting. The cells subsequently return to prefasting size when the mice are refed with basal or high levels of protein. Cell size is nearly doubled when mice maintained on low protein diets are given a basal diet, but increases only slightly when dietary protein is increased to a high level. The variations in liver cell size reflect the variations in the glycogen, fat and ribonucleoprotein in the liver.

5. Mitotic activity in the liver of the mouse during fasting and refeeding and during changes in dietary protein level is discussed in relation to the changes in liver protein under similar conditions which are reported by other investigators. The increase in mitotic activity in the liver appears to correspond in time to the increase in liver proteins.

6. The basophilic material in the cytoplasm of the hepatic cells which represents ribonucleic acid becomes reduced in amount in mice that are fasted and in those that are maintained on low protein diets. It is restored when such mice are given adequate or high levels of dietary protein. Its restoration coincides in time with maximum mitotic activity

in the liver. This is presented as further evidence of the association of mitotic activity in the liver with the synthesis of cytoplasmic proteins.

LITERATURE CITED

- ADDIS, T., L. J. POO AND W. LEW 1936a The quantities of protein lost by the various organs and tissues of the body during a fast. *J. Biol. Chem.*, **115**: 111-116.
- 1936b Protein loss from liver during a two day fast. *J. Biol. Chem.*, **115**: 117-118.
- 1936c The rate of protein formation in the organs and tissues of the body. I. After casein refeeding. *J. Biol. Chem.*, **116**: 343-352.
- BAKER, J. R. 1944 The structure and chemical composition of the Golgi element. *Quart. J. Micro. Sci.*, **85**: 1-71.
- BEARD, H. H. 1926 Studies in the nutrition of the white mouse. I. The normal growth and nutritive requirements. *Am. J. Physiol.*, **75**: 645-657.
- BERG, W. 1914 Über den mikroskopischen Nachweis der Eiweiss-speicherung in der Leber. *Biochem. Zeitschr.*, **61**: 428-433.
- 1920 Ueber funktionelle Leberzellstrukturen. I. Die Leberzelle von *Salamandra maculata* während des Zustandes der guten Ernährung und des Hungers. Die Einwirkung von Fütterung und von Beförderung der Gallenabsonderung bei Hungertieren. *Arch. f. mikr. Anat.*, **24**: 518-567.
- BRACHET, J. 1942 La localisation des acides pentosenucléiques dans les tissus animaux et les oeufs d'Amphibiens en voie de développement. *Arch. Biol.*, **55**: 207-257.
- BRACHET, J., AND R. JEENER 1944 Recherches sur des particules cytoplasmiques de dimensions macromoléculaires riches en acide pentosenucléique. I. Propriétés générales, relations avec hydrolases, les hormones, les protéines de structure. *Enzymologia*, **11**: 196-212.
- BRACHET, J., R. JEENER, M. ROSSEEL AND L. THONET 1946 Etude des variations de la teneur en acide-ribonucéique du foie au cours de divers états physiologiques. *Bull. Soc. Chem. Biol.*, **28**: 460-465.
- BURR, G. O., AND M. M. BURR 1930 On the nature and rôle of the fatty acids essential in nutrition. *J. Biol. Chem.*, **86**: 587-621.
- CASPIERSON, T. 1941 Studien über den Eiweissumsatz der Zelle. *Naturwiss.*, **29**: 33-43.
- CLAUDE, A. 1946 Fractionation of mammalian liver cells by differential centrifugation. II. Experimental procedures and results. *J. Exp. Med.*, **84**: 263-291.
- DAVIDSON, J. N., AND C. WAYMOUTH 1944 Tissue nucleic acids. 3. The nucleic acid and nucleotide content of liver tissue. *Biochem. J.*, **38**: 379-385.
- 1946 The nucleoproteins of the liver cell demonstrated by ultra-violet microscopy. *J. Physiol.*, **105**: 191-196.

- DEANE, H. W. 1944 A cytological study of the diurnal cycle of the liver of the mouse in relation to storage and secretion. *Anat. Rec.*, 88: 39-65.
- 1946 The basophilic bodies in hepatic cells. *Am. J. Anat.*, 78: 227-244.
- HARRISON, H. C., AND C. N. H. LONG 1945 The regeneration of liver protein in the rat. *J. Biol. Chem.*, 161: 545-557.
- JACKSON, C. M. 1925 The effects of inanition and malnutrition upon growth and structure. P. Blakiston's Son and Co., Philadelphia.
- JEENER, R., AND J. BRACHET 1941 Association, dans un même granule, de ferments et des pentosenucléoprotéides cytoplasmiques. *Acta Biol. Belg.*, 1: 476-481.
- KOSTERLITZ, H. W. 1944a Effect of dietary protein on liver cytoplasm. *Nature*, 154: 207-209.
- 1944b The effect of dietary protein on liver cytoplasm. *Biochem. J. (Proc.)*, 38: xiv-xv.
- 1947 The effects of changes in dietary protein on the composition and structure of the liver cell. *J. Physiol.*, 106: 194-210.
- KOSTERLITZ, H. W., AND I. D. CRAMB 1943 The effect of fasting on the protein content of the liver. *J. Physiol.*, 102: 18P.
- LAGERSTEDT, S. 1947 Investigations on the proteinaceous inclusions of the liver cell cytoplasm in ultraviolet light. *Acta Anat.*, 3: 84-94.
- LIGHTBODY, H. D., AND A. KLEINMAN 1939 Variations produced by food differences in the concentration of arginase in the livers of white rats. *J. Biol. Chem.*, 129: 71-78.
- LUCK, J. M. 1936 Liver proteins. I. The question of protein storage. *J. Biol. Chem.*, 115: 491-510.
- 1937 The liver proteins. *Perspectives in Biochemistry*. Edited by J. Needham and D. E. Green. 215-229. Cambridge University Press.
- MILLER, L. L. 1948 Changes in rat liver enzyme activity with acute inanition. Relation of loss of enzyme activity to liver protein loss. *J. Biol. Chem.*, 172: 113-121.
- MORPURGO, B. 1889 Sur les processus physiologique de neoformation cellulaire durant l'inanition aigue de l'organisme. *Arch. Ital. de Biol.*, 11: 118-133.
- PEARCE, E. L., H. E. SAUBERLICH AND C. A. BAUMANN 1947 Amino acids excreted by mice fed incomplete proteins. *J. Biol. Chem.*, 168: 271-282.
- PFUHL, W. 1938 Die mitotischen Teilungen der Leberzellen in Zusammenhang mit den allgemeinen Fragen über Mitose und Amitose. *Zeitsch. Anat. u. Entwicklungsgesch.*, 109: 99-133.
- STERNHEIMER, R. 1939 The effect of a single injection of thyroxin on carbohydrates, protein and growth in the rat liver. *Endocrinol.*, 25: 899-908.
- WILSON, J. W., AND E. H. LEDUC 1947 Mitotic rate in mouse liver following intraperitoneal injection of liver, kidney and egg yolk. *Anat. Rec.*, 97: 470-494.

MALFORMATION OF THE ATRIO-VENTRICULAR ENDOCARDIAL CUSHIONS OF THE EMBRYO PIG AND ITS RELATION TO DEFECTS OF THE CONUS AND TRUNCUS ARTERIOSUS

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FOURTEEN FIGURES

The literature on cardiac abnormalities deals almost exclusively with hearts from newborn and older individuals. The usual way of explaining a heart defect is to review the normal development and then to guess at what point the embryonic heart went astray. It occurred to me that I might study abnormal embryonic hearts, such as are old enough to show significant malformations, but still young enough to retain embryonic landmarks.

MATERIALS AND METHODS

I have to date examined 15,000 pig embryos and recovered 35 distinctly abnormal hearts. Nearly all of these came from embryos of from 20 to 50 mm length, which are roughly equivalent to human embryos of the third month. The hearts of both have attained practically adult configuration in the 20-mm embryo. My abnormal hearts are therefore sufficiently advanced to show significant cardiac malformations, but still young enough to preserve traces of embryonic structures that might help in understanding the malformation.

My routine procedure is to open the thorax of the embryo and to examine its heart for gross external abnormalities of

the ventricles and great vessels. Suspected hearts with lungs and adjacent thoracic wall attached were sketched and then sectioned serially. The heart should be cut transversely. About one in 6 suspected hearts turned out abnormal. The detailed study of an abnormal heart began with the preparation of a wax model of it.

OBSERVATIONS

When the 35 abnormal hearts were studied in serial section, 16 were found to exhibit defective fusion of the dorsal and ventral atrio-ventricular endocardial cushions. These 16 cases, which are the basis of this communication, are distributed as follows:

- 3 from embryos of 20 mm to 25 mm
- 7 from embryos of 25 mm to 30 mm
- 5 from embryos of 30 mm to 40 mm
- 1 from a 50-mm embryo

The fusion of the atrio-ventricular cushions should be well under way in the 10-mm embryo. Therefore, in all the 16 embryos that are the basis of this paper, the fusion and transformation of the cushions has been delayed long past the normal time. Various examples of non-fusion and mal-fusion of the dorsal and ventral atrio-ventricular cushions are represented. Three hearts show no fusion whatever. Most hearts show differing grades of retarded fusion, whereby the identity of the cushions is preserved.

When the hearts with defective atrio-ventricular cushions were studied as a group, one striking fact emerged. Every one of the 16 shows some external or internal malformation of the conus. Either the conus retains its primitive form, or projects as a noticeable promontory, or has a general swollen appearance. The aorta overrides the interventricular septum, or looks altogether into the right ventricle. The aortico-pulmonary septum stops above the semilunar valves, between them, or below them. The aorta and pulmonary artery are sometimes of equal caliber or some degree of stenosis may appear. Now it is quite possible that such a

correlation of atrio-ventricular cushion defects with conus and truncus defects may be due to my method of collecting, for I would have missed any atrio-ventricular defect that was not betrayed by some external deformity. Still the correlation is so high that it has led me to suspect that the one has something to do with the other.

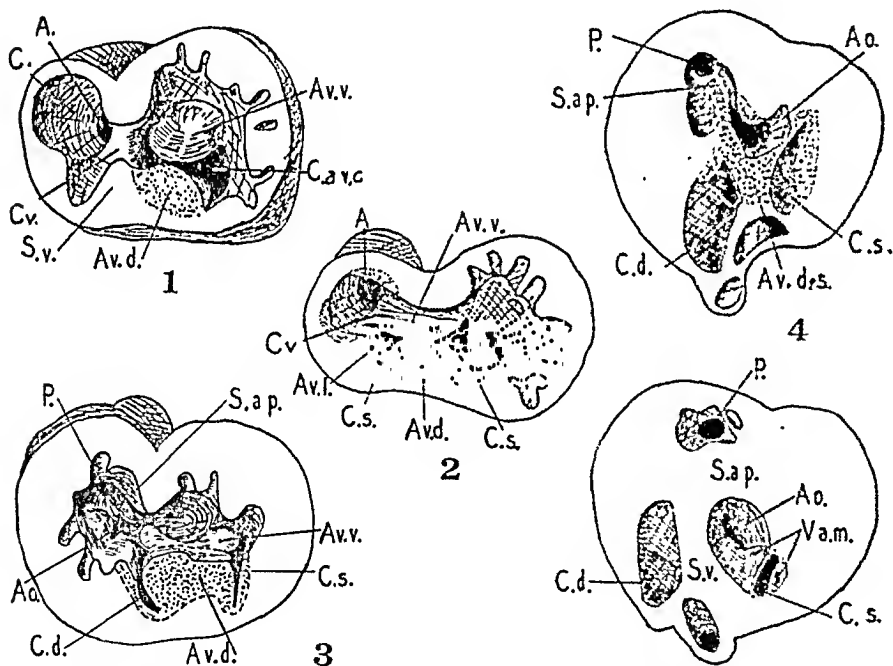
That the atrio-ventricular cushions are involved in the transformation of the conus and might affect its normal growth, will be appreciated when one reviews the normal development of the region.

The conus and truncus arteriosus of a normal 6-mm pig embryo heart are a simple tube that arises wholly from the right ventricle (fig. 1). The undivided atrio-ventricular canal of the same stage leads into the left ventricle only. Between the two ventricles there is a stout cono-ventricular ridge. In later stages the truncus arteriosus divides into aorta and pulmonary artery and the orifices of both shift to the left, so that the pulmonary orifice lies close to the interventricular septum and the aortic orifice lies just medial to the aortic cusp of the mitral valve (fig. 5). Two internal modifications are needed to permit such a migration. First, the cono-ventricular ridge is flattened out of existence (fig. 2). Secondly, after the dorsal and ventral atrio-ventricular canal cushions have fused (fig. 2), the ventral cushion is deeply excavated in its middle (fig. 3), so that only its two tubercles remain. The aortic orifice now can utilize the excavation in the ventral cushion (fig. 4) and drain the left ventricle. In addition the proximal part of the aortico-pulmonary septum now can reach the free edge of the interventricular septum and participate in the closure of the interventricular foramen (figs. 4 and 5).

Thanks to the work of Keith ('06, '33) it is now generally recognized that the reduction of the cono-ventricular ridge is a critical step in the development of the heart. Kramer ('42) assigns less weight to outright absorption and more to differential growth, but agrees with Keith on the significance of the disappearance of the ridge. Neither writer says much

ABBREVIATIONS (applying to all figures)

| | |
|---|------------------------------------|
| A., Truncus arteriosus | Cr., Crista supraventricularis |
| Ao., Aorta | Cr., Cono-ventricular ridge |
| At.d.,s., Right and left atrium | F.I., Interatrial foramen primum |
| Av.d.,v.,l., Dorsal, ventral and lateral atrio-ventricular endocardial cushions | F.v., Interventricular foramen |
| Br., Brachiocephalic artery | H., Bundle of His |
| C., Conus | M., Moderator band |
| C.av.c., Common atrio-ventricular canal | P., Pulmonary artery |
| C.d.,s., Right and left atrio-ventricular canal | S.a., Interatrial septum primum |
| ca., Canaliculus between endocardial cushions | S.ap., Aortico-pulmonary septum |
| | S.v., Interventricular septum |
| | V.a.m., Mitral valve |
| | V.a.t., Trienspid valve |
| | Vc.d.,s., Right and left ventricle |



Figs. 1-5 Interior views of reconstructions of hearts from normal pig embryos to show normal development of the dorsal and ventral atrio-ventricular cushions, the resorption of the cono-ventricular ridge and the migration of the aortic inlet. The apical part of each model has been removed. The reader looks into the base of the ventricles and into the conus.

- 1 from a 6-mm embryo. $\times 27$.
- 2 from a 7-mm embryo. $\times 22$.
- 3 from a 10-mm embryo. $\times 20$.
- 4 from a 14.5-mm embryo. $\times 15$.
- 5 from a 27-mm embryo. $\times 17$.

about the ventral atrio-ventricular cushion, yet it too is involved. As Odgers ('38) points out, the aortic channel must pass over the right tubercles of the atrio-ventricular cushions to reach the left ventricle. But if the large ventral cushion shown in figures 1 and 2 is not properly fused to the dorsal one and then deeply excavated as in figure 3, the migration of the aortic orifice must be halted or diverted. If halted, the aortic orifice is held in an overriding or "transposed" position. If the aorta does nevertheless migrate, it is thrust forward between the abnormal ventral atrio-ventricular cushion and the wall of the ventricle. The arrested migration of the aortic orifice would in turn deflect the aortico-pulmonary septum and prevent the closing of the interventricular foramen. In general, a proper fusion of the atrio-ventricular cushions, and a deep excavation of the ventral one are as important as the disappearance of the cono-ventricular ridge for the proper development of the aortic orifice into the left ventricle. An arrest in the growth or in the secondary resorption of the atrio-ventricular cushions may therefore be expected to affect the development of the conus and the aortic and pulmonary orifices.

Serial sections of abnormal hearts are fascinating objects for study, but a detailed description of them makes tedious reading. It is better to bring out the significant defects and the bearing of these upon well known forms of cardiac anomalies familiar from postnatal studies. Taking everything into account the abnormal pig hearts under consideration fall into three groups: (a) those in which the pulmonary artery and aorta are of equal caliber, (b) those in which the aorta is definitely narrowed, and (c) those showing pulmonary stenosis. The grouping is admittedly question-begging as far as causation is concerned. The reader may reserve judgment on its usefulness until the end of the paper.

Group A. Aorta and pulmonary artery of equal caliber

Case 1, No. 881, 28 mm

Case 2, No. 904, 30 mm

Case 3, No. 860, 28 mm

Case 1, from a 28-mm embryo (fig. 6) shows externally a rather primitive conus that is still marked off from the right ventricle. An incidental anomaly is a small right aortic arch. Within the heart the dorsal and ventral atrio-ventricular canal cushions are quite distinct (fig. 6c). They are joined to the interatrial septum I above and the interventricular septum beneath (fig. 6b, d). The only other connection is a

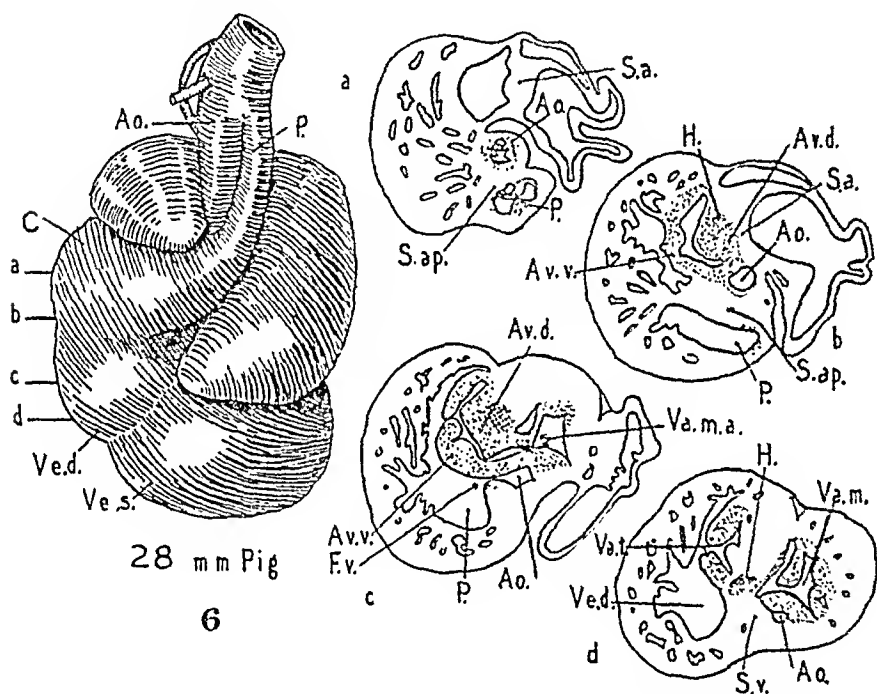


Fig. 6 Model and transverse sections of an abnormal heart, case 1, from a 28-mm pig embryo. Alberta Embryological Collection no. 881. The stippled areas are endocardial cushion tissue. Sections $\times 12$.

a, through the semilunar valves.

b and c, through the endocardial cushions of the atrio-ventricular canal.

d, through the tricuspid and mitral valves.

trace of fusion at the future aortic cusp of the mitral valve (fig. 6c). The aorta and pulmonary artery are normal, with properly orientated semilunar valves (fig. 6a). The aortico-pulmonary septum is well developed. The interventricular foramen is very nearly closed. The interatrial foramen II is

wide open in this and all other hearts described, as is to be expected in fetal hearts.

The atrio-ventricular cushions of this 28-mm pig heart have not progressed beyond the condition found in 10-mm pigs. The nonfusion of the cushions has not, however, prevented the normal migration of the aortic orifice to the left,

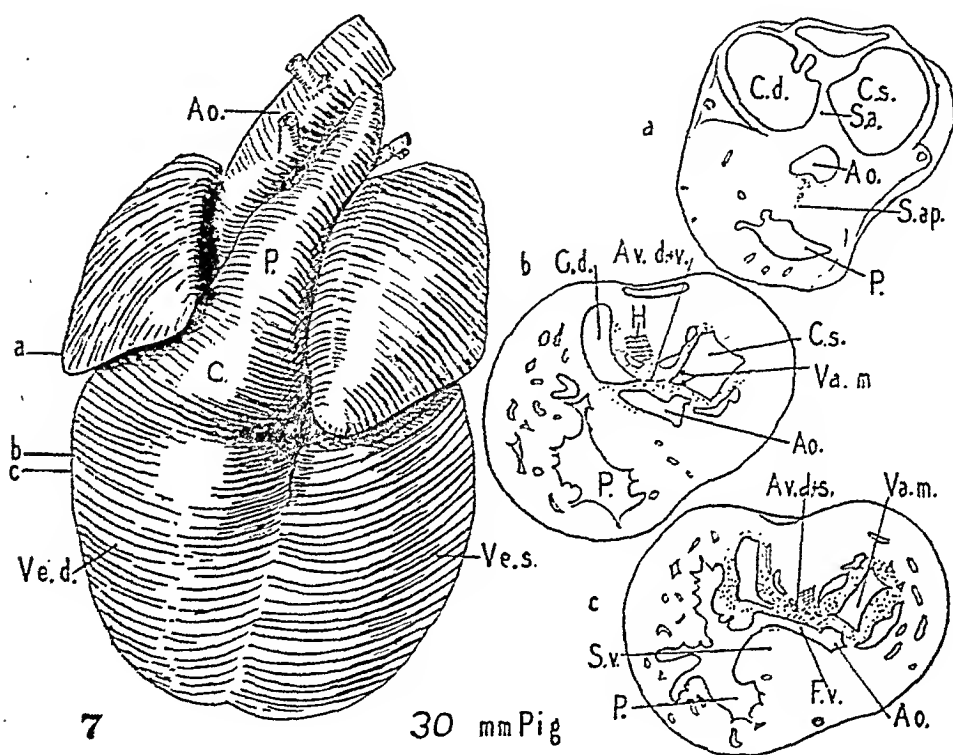


Fig. 7 Model and transverse sections of an abnormal heart, case 2, from a 30-mm pig embryo, A.E.C. no. 904. The stippled areas are endocardial tissue. Sections $\times 10$.

a, through conus.

b and c, through atrio-ventricular endocardial cushions.

nor interfered with the downward growth of the proximal part of the aortico-pulmonary septum, but the persisting cushions have pushed the aortic orifice ventrally and compressed it against the heart wall (fig. 6b, c, d). The orifice is indeed smaller than the aorta above it, and the heart might have developed aortic stenosis had the embryo lived longer.

Case 2, from a 30-mm embryo (fig. 7) shows a conus quite prominent and projecting, but more absorbed into the right ventricle than in case 1. The right ventricle is somewhat larger than the left and thicker walled. The aorta and pulmonary artery are normal, however, with normally orientated semilunar valves.

Within the heart the dorsal and ventral atrio-ventricular cushions are much like those in case 1, but they have gone one step further and fused in their central parts (fig. 7b, c). The tricuspid valve cusps are nondescript. The aortic mitral cusp is partly cleft (fig. 7b, c). The aortico-pulmonary septum does not extend so far into the heart as in case 1, and a considerable interventricular foramen remains (fig. 7c).

The aortic orifice overrides the ventricular septum, and looks more into the left ventricle than the right (fig. 7b). The normal migration of the aorta has been somewhat hindered and its orifice into the left ventricle is squeezed between the ventral atrio-ventricular cushion and the heart wall (fig. 7b, c).

Case 3, from a 28-mm embryo (fig. 8) shows a swollen conus which is part of a much enlarged right ventricle. The aorta and pulmonary artery are of equal and normal caliber, but the aorta is definitely "transposed." Both sets of semilunar valves are rotated counterclockwise (fig. 8a). Within the heart, the atrio-ventricular cushions are quite distinct and not fused to each other (fig. 8b, c, d). They are joined, however, to the interatrial septum above and the interventricular septum beneath. The aortico-pulmonary septum stops a little below the semilunar valves, leaving a wide open interventricular foramen.

The orifice of the aorta overrides the ventricular septum, the vessel arising more from the right ventricle than the left (fig. 8b, c, d). What should be the normal aortic orifice from the left ventricle is a cleft squeezed between the ventral atrio-ventricular cushion and the heart wall (fig. 8d). The persisting ventral atrio-ventricular cushion has obstructed

the free migration of the aortic orifice and crowded the orifice against the ventricular wall.

All three hearts of Group A are from embryos of 28–30 mm, but they have atrio-ventricular cushions normally found at the stage of 10–12 mm. The aorta and pulmonary artery

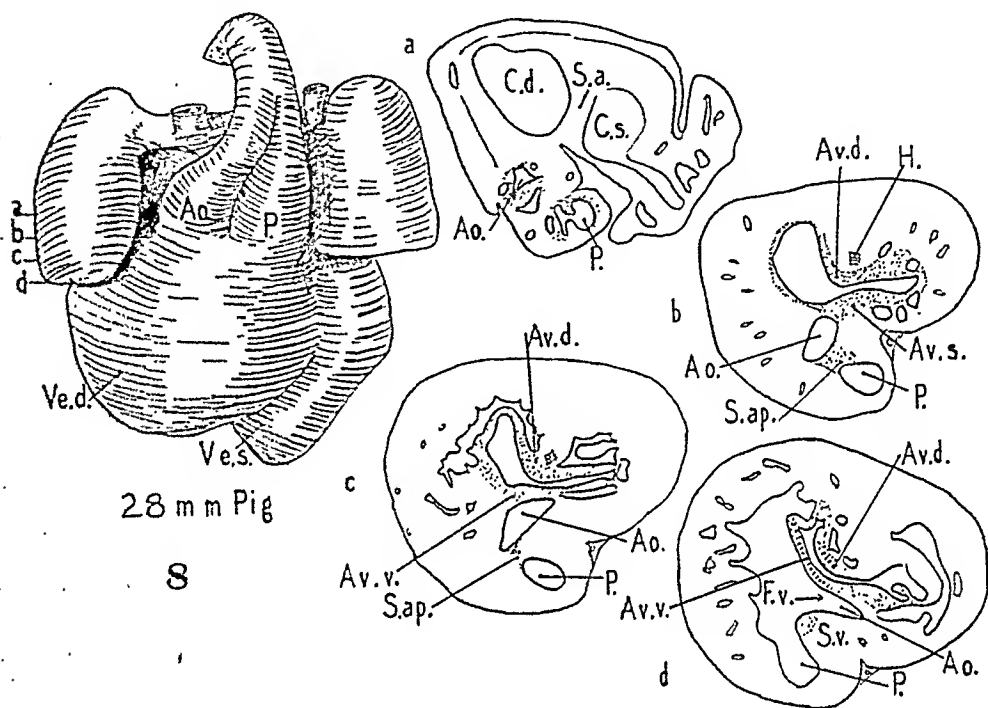


Fig. 8 Model and transverse sections of an abnormal heart, case 3, from a 28-mm pig embryo, A.E.C. no. 860. The stippled areas are endocardial tissue. Sections $\times 10$.

a, through the semilunar valves.

b, c and d, through the atrio-ventricular endocardial cushions.

are of normal caliber, but the normal aortic orifice is displaced, and with the displacement there is a corresponding incompleteness of the aortico-pulmonary septum. Now such a displacement of the aorta does occur as an independent defect, and Bremer ('42) has suggested a plausible explanation for it. For the hearts just described, however, it seems reasonable to attribute the displacement of the aortic orifice to an abnormal atrio-ventricular cushion, which should have

fused with its fellow, and then been reabsorbed in its central part, allowing the aorta to migrate to the left.

Cases 1 and 2 are good embryonic examples of the 50 or so known cases of postnatal hearts with a common atrio-ventricular ostium, recently reviewed by Rogers and Edwards ('48). Case 3 is an embryonic example of Eisenmenger's complex. It corresponds fairly well to the adult case described by Baumgartner and Abbott ('29), which presented the characteristic features of the complex: absence of pulmonary stenosis, prominence of the pulmonary conus, hypertrophy of the right ventricle, overriding aorta and patent interventricular foramen. The authors make the additional and significant observation that the left end of the septal tricuspid cusp passes through the interventricular septal defect and becomes continuous with the aortic cusp of the mitral valve and is also attached to the aortic ring, an arrangement which plainly suggests an early malformation of the ventral atrio-ventricular cushion. While I do not think that all cases of Eisenmenger's complex arise from defective development of the endocardial cushions, such a maldevelopment could well be one effective primary cause of the complex.

Group B. Definite stenosis of the aorta

Case 4, No. 859, 25 mm

Case 5, No. 833, 50 mm

Case 6, No. 842, 35 mm

The striking feature of case 4, from a 25-mm embryo, is the extreme stenosis of the aorta (fig. 9). The vessel is rudimentary as far as the brachiocephalic (innominate) artery, so that the pulmonary artery with the ductus arteriosus makes up the first part of the aortic arch. Internally the significant feature is the complete lack of fusion between the dorsal and ventral atrio-ventricular cushions (fig. 9f). Both cushions are attached as usual to the interventricular septum beneath (fig. 9g), but above them the interatrial septum is vestigial and there is a large interatrial foramen

I. The aortico-pulmonary septum stops short of closing the interventricular foramen.

The relation of the aorta to the ventral atrio-ventricular cushion is significant. The aorta at the brachiocephalic artery is of capillary size (fig. 9a). It is somewhat larger at the semilunar valve level (fig. 9c). Below the valves the aorta is compressed by an overgrowth of the ventral atrio-ventricular cushion (fig. 9d) which again constricts its lumen. The aortic orifice is pushed forward between the ventral cushion and the heart wall (fig. 9e).

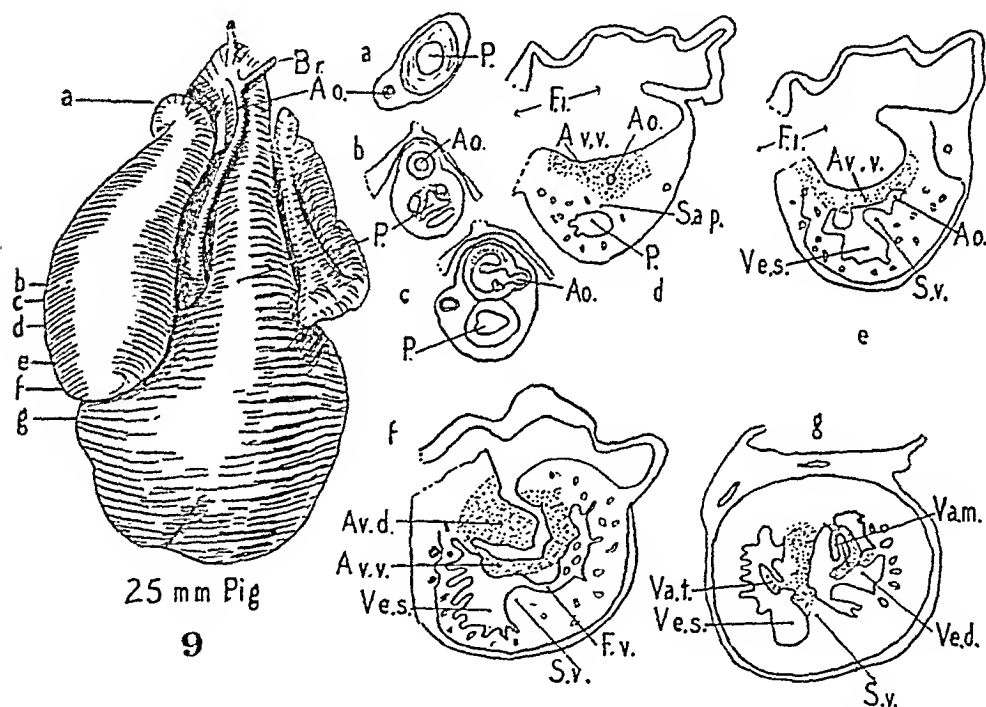


Fig. 9 Model and transverse sections of an abnormal heart, case 4, from a 25-mm pig embryo, A.E.C. no. 859. The stippled areas are endocardial tissue. Sections $\times 12$.

a, through the great vessels just proximal to the origin of the pulmonary arteries.

b, through the semilunar valves of the aorta.

c, through the semilunar valves of the pulmonary artery.

d and e, through the undivided atrium and the ventral atrio-ventricular cushion.

f, through the dorsal and ventral atrio-ventricular cushions.

g, through the tricuspid and mitral valves.

The cusps of the semilunar valves are normally and symmetrically fashioned, indicating that the original division of the truncus arteriosus into aorta and pulmonary artery was normal. But below the valves, where the proximal aortico-pulmonary septum meets the ventral atrio-ventricular cushion (fig. 9d), there is an overgrowth or untimely persistence of endocardial tissue which constricts the aortic orifice. The

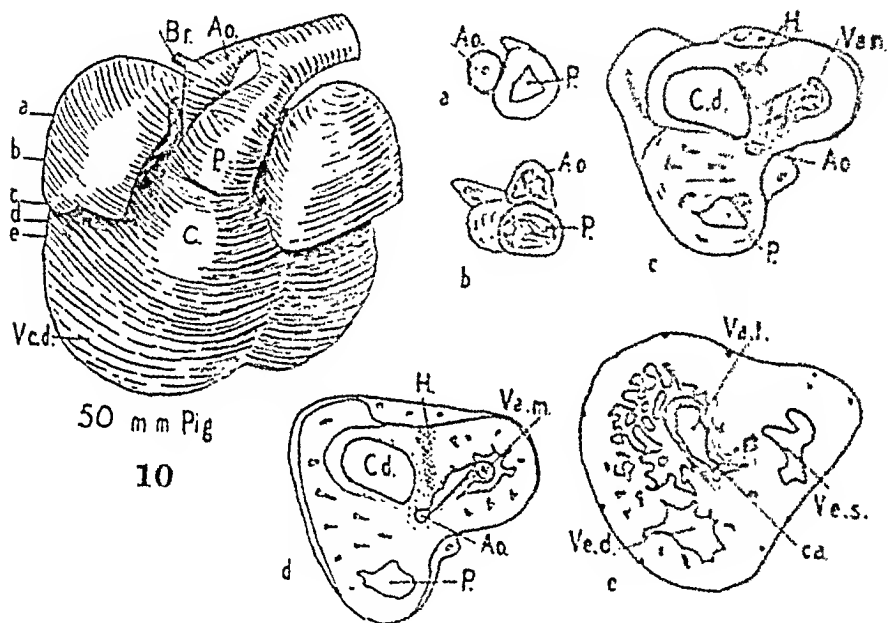


Fig. 10 Model and transverse sections of an abnormal heart, case 5, from a 50-mm pig embryo. A.E.C. no. 833. The stippled areas are endocardial tissue. Sections $\times 7\frac{1}{2}$.

- a, through great vessels just proximal to the origin of the pulmonary arteries.
- b, through the semilunar valves.
- c, d and e, through the atrio-ventricular cushions.

constriction reduced the flow through the aorta, and the vessel lagged behind in its growth, as does the ischiadic artery in the normal embryo, when blood is diverted from that vessel. Case 4 is possibly a step beyond case 1 (cf. fig. 6b, c, with fig. 9d, e).

Case 5, from a 50-mm embryo (fig. 10) shows aortic stenosis slightly less advanced than case 4. The aorta is narrow

throughout and has diminutive semilunar valve cusps (fig. 10a, b, c, d). The pulmonary artery and the ductus arteriosus form the first part of the aortic arch.

Internally the atrio-ventricular cushions show defective fusion and growth. The tricuspid orifice is of normal size but the mitral valve is stenosed. An irregular strand of connective tissue, the remains of the endocardial cushions of the atrio-ventricular canal, extends from one valve to the other and surrounds and constricts the aortic inlet (fig. 10c, d, e). The strand is perforated at its tricuspid end by a canaliculus which marks the line of fusion of the endocardial cushions. The aortico-pulmonary septum is fully developed and no interventricular foramen remains.

The irregular fusion and development of the atrio-ventricular cushions has obstructed the aortic orifice and hindered its proper migration. It is likely that the aorta and pulmonary artery were normally formed and of equal size in the beginning. But obstruction to blood flow into the aorta slowed down its development. The extreme narrowing of the aorta proximal to the ductus arteriosus is an added effect of fetal coarctation. Whether the mitral valve stenosis is primary or secondary cannot be said.

Case 6, from a 35-mm embryo (fig. 11) has great vessels much like those of case 5. The aorta exhibits pronounced fetal coarctation, a stenosed and tapering lumen, and undersized but symmetrical semilunar valves. As with case 5, the aortico-pulmonary septum is completed, and no interventricular foramen remains.

The tricuspid and mitral valves are connected by a substantial strand of endocardial cushion tissue which is embedded in muscle (fig. 11c). The strand blocks all interventricular communication and shuts the aorta off from the left ventricle. The aortic orifice has been held in its early right sided or "transposed" position and receives blood from the right ventricle only, and that through a narrow cleft between the crista supraventricularis and the medial papillary muscle of the tricuspid valve (fig. 11b, c).

The abnormal persistence of endocardial tissue of the dorsal and ventral cushions has prevented the migration of the aortic orifice from the right to the left ventricle, and kept the orifice in an abnormal location, where it can be squeezed

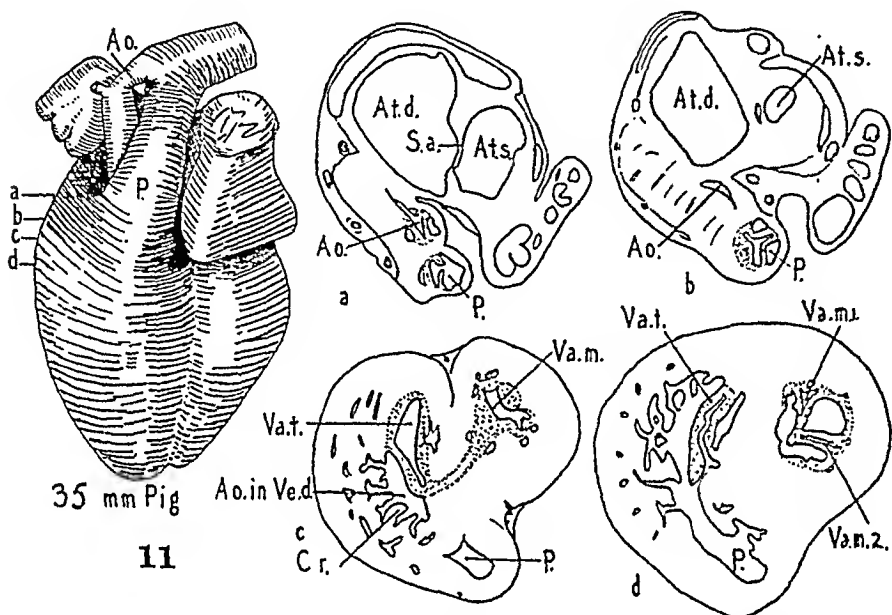


Fig 11 Model and transverse sections of an abnormal heart, case 6, from a 35-mm pig embryo, A.E.C. no. 842. The stippled areas are endocardial tissue. Sections $\times 10$.

a, through semilunar valves.

b, through the abnormal origin of the aorta from the right ventricle, and through the narrowed left atrio-ventricular canal above the mitral valve.

c, through the endocardial strand, formed from the fused atrio-ventricular cushions, that continues to connect the tricuspid and mitral valves.

d, through the mitral valve. Its two cusps are fused in their centers, dividing the single orifice into two.

between the crista supraventricularis and the interventricular septum each time the heart contracts. The division of the truncus arteriosus into aorta and pulmonary artery was probably normal, but the aorta later lagged behind because of the abnormally restricted blood flow through it. Fetal coarctation increased the aortic handicap.

The left ventricle has no outlet. The left atrio-ventricular canal is narrow (fig. 11b) and the mitral cusps are joined in their central parts, so that the mitral valve is doubled (fig. 11d). This is a very rare form of heart abnormality and the first recorded embryonic example of it (note 1).

The hearts of group B indicate that an abnormal ventral atrio-ventricular cushion may be an effective cause of aortic stenosis. The cushion may reduce the aortic orifice by surrounding and constricting it, or by displacing it and squeezing it against the heart wall. It is likely that the original division of the truncus arteriosus into aorta and pulmonary artery was normal and equal, for the three cusps of the semilunar valves are symmetrical and of equal size. Cutting off of blood flow through the aorta caused the vessel and its valves to lag behind the pulmonary artery.

In cases 4 and 5 the endocardial tissue surrounds the aortic inlet. Had the embryo hearts continued to develop, the endocardial tissue would have formed the annular obstruction found in postnatal cases of subaortic stenosis. One would expect the postnatal "ring" to preserve occasionally some connection with the nearby mitral cusp, since both are derived from the same endocardial cushion. This does occur. In the well known case of Keith ('24, fig. 3) the subaortic ring is continuous with the mitral valve cusp. In the case given by Taussig ('47, fig. 134) there is an anomalous insertion of the anterior or aortic cusp of the mitral valve into the inner aortic wall beneath the semilunar valve, just as one might anticipate if the endocardial cushion were the cause of the malformation.

However, one should not push this explanation of general aortic stenosis too far. Any arrangement that obstructs the aortic blood flow may cause stenosis. Many ordinary instances of aortic stenosis are probably cases of overextended fetal coarctation. Writers are prone to appeal to intra-uterine infection as an active cause of aortic stenosis. The endocardial tissue of my pig hearts appears to be quite normal.

Group C. Stenosis of the pulmonary artery

Case 7, No. 852, 30 mm

Case 8, No. 858, 27 mm

Case 9, No. 853, 35 mm

The striking external feature of case 7 from a 30-mm embryo is the long, tortuous and primitive conus (fig. 12), which is larger than the right ventricle. The left ventricle is enlarged. The pulmonary artery is slightly stenosed (fig. 12a). The semilunar valves of the great vessels are rotated counterclockwise, and the aortic orifice overrides the inter-ventricular septum (fig. 12b). The aortico-pulmonary septum ends just below the valves.

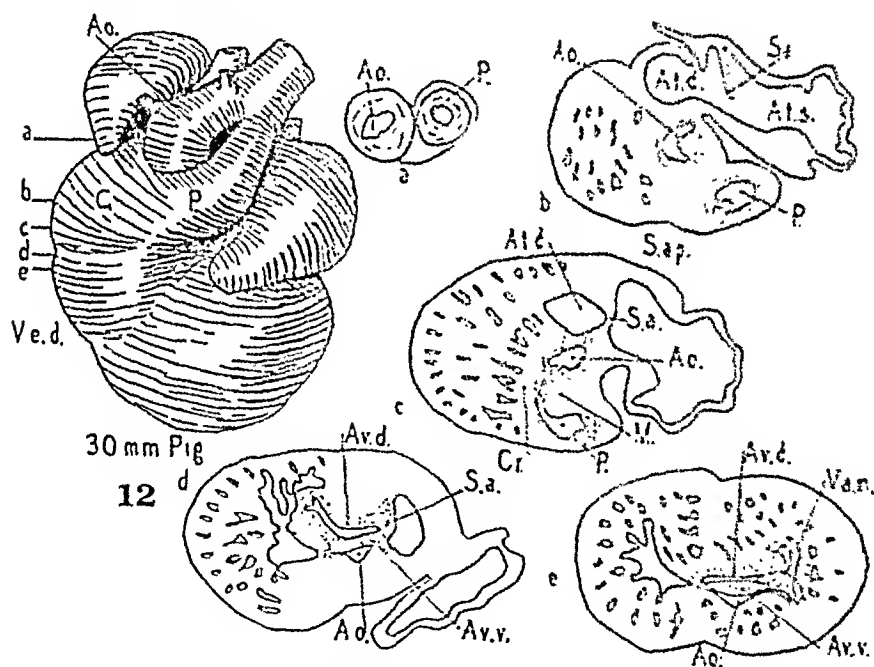


Fig. 12 Model and transverse sections of an abnormal heart, case 7, from a 30-mm pig embryo, A.E.C. no. 852. The stippled areas are endocardial tissue. Sections $\times 12$.

a, through the great vessels just proximal to the origin of the pulmonary arteries.

b, through the semilunar valves.

c, through the conus just below the arrested aortico-pulmonary septum.

d and e, through the atrio-ventricular endocardial cushions.

The dorsal and ventral atrio-ventricular cushions are primitive (fig. 12d, e). The interatrial septum meets them rather far to the left (fig. 12d). The tricuspid cusps are roughly marked out, and the two parts of the aortic cusp of the mitral valve have just begun to fuse (fig. 12e). The aortic orifice is squeezed between the ventral cushion and the heart wall (fig. 12d, e).

With such a cramped aortic orifice the heart would seem destined for aortic stenosis. But the aortico-pulmonary septum ends so high that the aorta can receive blood from the right ventricle as well as the left. The pulmonary artery on the contrary has a tortuous course, with one kink below the semilunar valve and another where the pulmonary conus should have widened into the right ventricle. The horizontal stretch between the two kinks lies between a strong crista supraventricularis and the moderator band within, and the ventricular wall without, and is probably constricted at systole. The aorta lies deep, with access to both ventricles, and is a readier exit for blood than the pulmonary artery. The pulmonary artery has begun to lag behind because of reduced blood flow through it.

Case 8, from a 27-mm embryo, shows a conus not so primitive as the preceding but still prominent, projecting, and marked off from the small right ventricle (fig. 13). The pulmonary artery is perhaps a trifle undersized (fig. 13a). The great vessels are rotated counterclockwise at the semilunar valve level (fig. 13b) so that the aorta overrides the interventricular septum. The aortico-pulmonary septum ends at the level of the semilunar valves (fig. 13c), and the aorta can receive blood from both ventricles. The transformation of the distal conal endocardial ridges into semilunar valve cusps is imperfect (fig. 13b). Below the valves, the proximal endocardial ridges which should have formed the septum do not appear as such. The endocardial tissue simply lines the undivided conal lumen and forms a broad adhesion that narrows the pulmonary inlet (fig. 13d).

The dorsal and ventral atrio-ventricular cushions are partly fused (fig. 13e); the two moieties of the aortic cusp of the mitral valve are still distinct. In this heart the persisting atrio-ventricular cushions are still large enough to prevent the proper migration of the aorta, which in turn

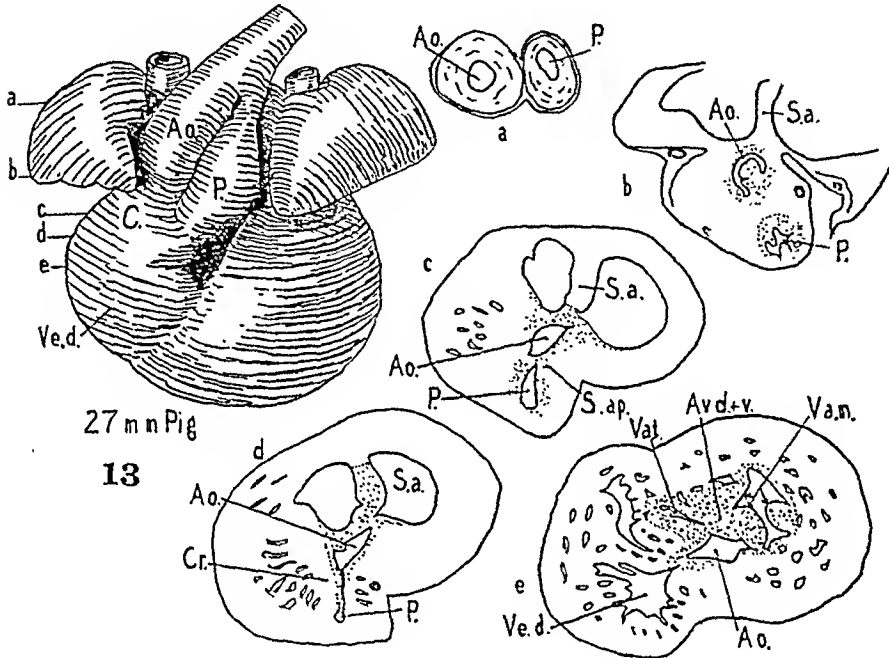


Fig. 13 Model and transverse sections of an abnormal heart, case 8, from a 27-mm pig embryo, A.E.C. no. 858. The stippled areas are endocardial tissue. Sections $\times 12$.

a, through the great vessels just proximal to the origin of the pulmonary arteries.

b, through the semilunar valves.

c, through the lower edge of the aortico-pulmonary septum.

d, through the pulmonary conus. The conus arteriosus is narrowed by endocardial fusion.

e, through the atrio-ventricular endocardial cushions.

has kept the pulmonary artery in a prominent and projecting position. The aorta, thanks to its central position and the wide-open interventricular foramen, can drain both ventricles. As in the previous case, I suspect a positive obstruction of the pulmonary artery. The valve region of the pulmonary

artery is thrust ventrally by the misplaced aorta, and the conus arteriosus of the pulmonary artery leaves the main ventricle like the spout of a coffee pot. The conus arteriosus is bounded within by a strong crista supraventricularis. At systole the crista could approximate the sides of the conus arteriosus. The broad adhesion of endocardial tissue which partly closes the pulmonary lumen below the valves (fig. 13d) indicates that some such approximation is taking place. Denied the stimulus of a normal blood flow, the pulmonary artery is lagging behind in growth, and might have become definitely stenosed had the embryo lived longer.

Case 9, from a 35-mm embryo (fig. 14), shows undeniable pulmonary stenosis. The conus is projecting, although less so than in the two preceding cases. The pulmonary artery is quite narrow (fig. 14a). The aortico-pulmonary septum ends at the semilunar valve level (fig. 14c) leaving a large interventricular foramen. There is considerable counter-clockwise rotation of the great vessels at the valve level so that the aorta arises to the right of the interventricular septum (fig. 14b, c). The aorta can receive blood from both ventricles. The pulmonary valves are defective (fig. 14c). Below the valves, the pulmonary conus is compressed and filled with endocardial adhesions (fig. 14d, e).

The atrio-ventricular cushions have undergone delayed and imperfect fusion. The boundary between the two cushions is marked by a seam and a tiny canal that runs from the tricuspid valve to the interventricular canal (fig. 14c, d, e).

My interpretation of case 9 is as follows. The belated and imperfect fusion together with inadequate resorption of the atrio-ventricular cushions has kept the aorta to the right and pushed the pulmonary artery ventrally so that the pulmonary conus retains the primitive projecting form. The aorta can receive blood freely from both ventricles, thanks to the defective proximal part of the aortico-pulmonary septum. It lies deep where it is not greatly disturbed by systole. The pulmonary artery has a vulnerable position and

is readily compressible between the crista supraventricularis and the heart wall.

Cases 7, 8, and 9 are progressive stages in the stenosis of the pulmonary artery. The artery in each case is a dwarfed or stunted embryonic vessel that has been denied the stimulus of a normal blood flow.

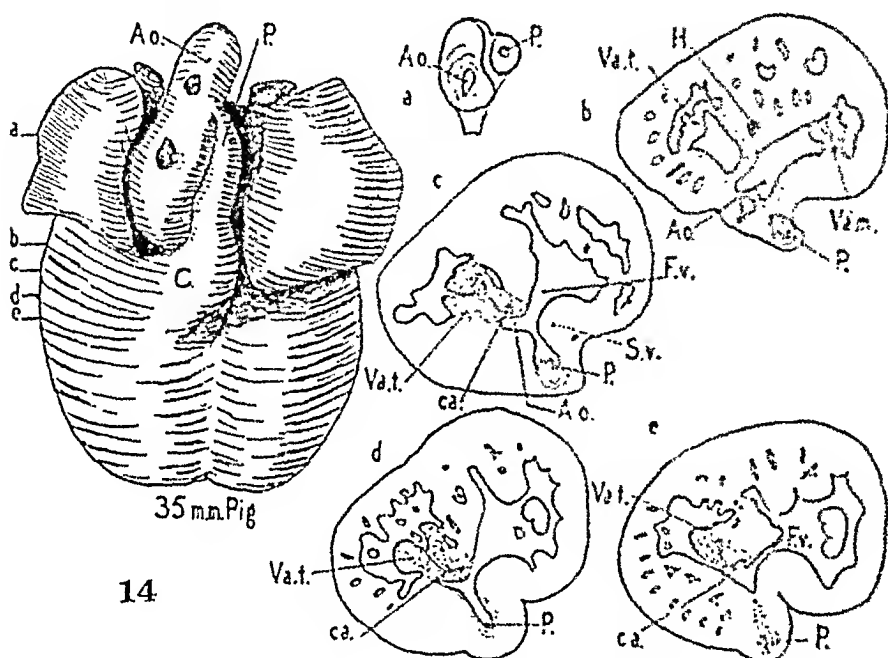


Fig. 14 Model and sections of an abnormal heart, case P, from a 35-mm pig embryo, A.E.C. no. 853. The stippled areas are endocardial tissue. Sections $\times 12$.

a, through the great vessels just proximal to the origin of the pulmonary arteries.

b, through the semilunar valves.

c, d and e, through the atrio-ventricular cushions and the pulmonary conus. Note the canaliculus that marks the fusion line of the cushions and the general endocardial fusion that obstructs the pulmonary conus.

DISCUSSION AND CONCLUSIONS

The embryonic pig hearts described above are good evidence for Keith's thesis that the conus is a critical part of the developing heart and the site of many of its graver anomalies. Keith treated the conus as a separate entity. It is of course

true that the conus may misbehave alone. But in a surprising number of hearts the defects of the conus are linked with defects of the atrio-ventricular cushions in such a way as to suggest the latter as the primary cause. McCullough and Wilbur ('44) have advanced the same idea after a study of postnatal human hearts. The evidence I have given in this paper all goes to show that defects of the atrio-ventricular cushions, hitherto treated as isolated affairs and effecting only the atrio-ventricular valves, have a wider significance.

Maldevelopment of the atrio-ventricular canal cushions may seem too rare to be the predisposing factor of the comparatively common defects of the conus and truncus arteriosus. It is true that larger defects such as a common atrio-ventricular ostium are not frequent. But there are probably many cases of smaller defects which are overlooked. Such a tiny defect as the canaliculus given in cases 5 and 9 has been found in one postnatal heart by Conforth and recorded by McCullough and Wilbur ('44). Were the atrio-ventricular valves carefully gone over in all cases of conal abnormalities, more defects that betray delayed and imperfect fusion of the cushions might be found.

The embryo pig hearts suggest a new answer to two old questions: Why do the pulmonary artery and the aorta become stenosed, and why is pulmonary stenosis much more frequent than aortic?

The classic explanation for stenosis is some variation of Rokitansky's (1875) schema, which postulates a swerving of the aortico-pulmonary septum and an unequal division of the truncus arteriosus. Rokitansky's schema antedates modern heart embryology and is hard to read into actual embryos, but his basic idea is still widely accepted. It is well expressed by Patten ('46).

Anomalies may result from the abnormal partitioning of the primitive ventral aortic trunk to form the ascending aorta and the pulmonary artery. The division may occur in such a manner that there is a marked narrowing of the aorta combined with a pulmonary artery which is unusually large, or the reverse condition may exist. (p. 672.)

No one, so far as I know, has found an embryonic heart in which such abnormal partitioning is taking place. I have several embryo pig hearts showing an arrest of the aortico-pulmonary partition at various levels, but none gives any evidence of unequal division. It seems to me more probable that the division of the primitive aorta is generally into approximately equal parts, so that the aorta and pulmonary artery start with equal calibers. Then some cardiac anomaly deflects blood from one vessel into the other. The slighted vessel lags behind in consequence, just as do many other embryonic vessels in the normal growth of the embryo. Stenosis and atresia should be regarded as the secondary results of some other cardiac disorder of structure and function.

The fundamental defect in both aortic and pulmonary stenosis is the retention of the characteristics of the early conus beyond their time. Such a retention probably has several causes, of which the defective development of the atrio-ventricular cushions is only one. But whatever the cause, the heart with such a conus shows three well known characters: (a) an overriding or right-sided aorta, (b) a projecting superficial pulmonary inlet, and usually (c) an open inter-ventricular foramen.

So long as both vessels have normal blood supply from the heart and are not otherwise obstructed (e.g., by coarctation) both vessels remain equal in caliber.

Occasionally the malformation of the atrio-ventricular cushions constricts the aortic inlet or squeezes it against the heart wall. Flow of blood into the aorta drops off and the vessel fails to keep pace in growth. Such circumstances are infrequent and aortic stenosis is uncommon. The aorta arises from the center of the heart base in even grossly abnormal hearts, where it has free communication with both ventricles and is not easy to shut off.

In most cases the scalés are tipped the other way. The pulmonary orifice, always superficial, is even more so in all

hearts with an abnormal conus. The more superficial the orifice is, the more liable it is to compression between the crista supraventricularis within and the cono-spiral muscle of the heart wall. There is much greater likelihood of obstruction of the pulmonary orifice. One would expect pulmonary stenosis to be more common, as is indeed the case.

SUMMARY

1. The author has examined 15,000 pig embryos and found 35 abnormal hearts. In half of these the external malformations of the conus and great vessels were associated with malformations of the atrio-ventricular canal cushions within.

2. A review of the normal development shows that a defective formation of the atrio-ventricular cushions could affect the proper development of the conus and the great vessels.

3. Nine embryo pig hearts, of an age equivalent to the human fetus of the third month, are described in detail. Three show the great vessels of equal caliber, three show aortic stenosis, and three pulmonary stenosis.

4. One common cause underlies all three groups. The defective development of the atrio-ventricular canal cushions prevents the proper migration of the aortic orifice to the left ventricle. If the aorta and the pulmonary artery continue to receive an adequate blood supply, the vessels grow to normal caliber. Occasionally the endocardial tissue surrounds and constricts the aortic orifice and produces aortic stenosis. More often the pulmonary loses blood to the more favorably situated aorta, and is subject to compression in its exposed position. Stenosis of the pulmonary artery follows.

5. Stenosis of either vessel is the dwarfing or stunting of an embryonic vessel that has been deprived of the stimulus of an adequate blood supply. It is an abnormal example of a normal phenomenon in the development of the vascular system.

6. While a deviation of the conus septum is not impossible as the primary cause of stenosis, other hitherto unsuspected defects within the heart may produce the same results. They should be considered when postnatal hearts are interpreted.

I wish to record my great obligation to the Swift Canadian Company, for the facilities freely given me to obtain material for this study, and to Mr. A. G. Fairall who prepared the material.

Note 1. A case of the rare double mitral orifice in the adult yak has been carefully studied by Wimsatt and Lewis ('48), and the two authors record independently their views on the genesis of the defect. Lewis attributes the accessory mitral orifice to the hole that would remain in the aortic cusp if there were only partial fusion between the two atrio-ventricular cushions. The aortic mitral cusp in my case 1 (fig. 6c) and case 7 (fig. 12e) would be perforated if fusion went no further, and the perforation could become an accessory mitral orifice. The other explanation of the accessory orifice is that of Hartmann ('37) and favored by Wimsatt; they ascribe the duplication to a fusion of the two mitral cusps. My case 6 (fig. 11d) is an example of such fusion. In my pig heart, however, the axes of the two orifices converge medially, while in the yak and human examples they converge laterally. The explanation favored by Lewis seems more probable, but that of Hartmann and Wimsatt is not impossible.

LITERATURE CITED

- BAUMGARTNER, S. A., AND M. E. ABBOTT 1929 Interventricular septal defect with dextroposition of aorta and dilation of the pulmonary artery ("Eisenmenger Complex") terminating by cerebral abscess. *Am. J. Med. Sci.*, 177: 639-647.
- BREMER, J. L. 1942 Transposition of the aorta and the pulmonary artery. *Arch. of Pathol.*, 34: 1016-1030.
- KEITH, A. 1906 Malformations of the bulbus cordis. *Studies in Pathology*, Aberdeen University Studies, no. 21: 57-74.
- 1924 Fate of the bulbus cordis in the human heart. Schorstein lecture. *Lancet*, II: 1267-1273.
- 1933 Human Embryology and Morphology. Arnold and Co. London.

- KRAMER, T. C. 1942 The partitioning of the truncus and conus and the formation of the membranous portion of the interventricular septum in the human heart. *Am. J. Anat.*, 71: 343-370.
- MCCULLOUGH, A. W., AND E. L. WILBUR 1944 A defect of the endocardial cushion development as a source of cardiac anomaly. *Am. J. Pathol.*, 50: 321-328.
- ODGERS, P. N. B. 1938 The development of the Pars Membranacea Septi in human heart. *J. Anat.*, 72: 247-259.
- PATTEN, B. M. 1946 *Human Embryology*. Blakiston, Philadelphia.
- ROGERS, M., AND J. E. EDWARDS 1948 Incomplete division of the atrio-ventricular canal with patent interatrial foramen primum. *Am. Heart J.*, 36: 23-54.
- ROKITANSKY, C. F. 1875 *Die Defecte der Scheidewände des Herzens*. Wien.
- WIMSATT, W. A., AND F. T. LEWIS 1948 Duplication of the mitral valve and a rare apical interventricular foramen in the heart of a yak calf. *Am. J. Anat.*, 58: 67-109.

THYMIC INVOLUTION AND REGENERATION IN THE ALBINO RAT, FOLLOWING INJECTION OF ACID COLLOIDAL SUBSTANCES

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TWENTY-SIX FIGURES

INTRODUCTION

In the studies here reported, an attempt has been made to follow changes in the thymus of the rat after intraperitoneal injections of Chlorazol black E or colloidal mercuric sulfide. These materials are acid colloids of relatively low toxicity. Under conditions of the experiment, the thymus undergoes involution and, if sufficient time is allowed, regeneration eventually reestablishes the organ into essentially normal state. These changes have been followed histologically, and special attention has been directed to reactions of the thymocyte and of the thymic epithelium. In addition, studies have been made on production and activities of macrophages in this organ.

The thymus has been the subject of many investigations; the literature was estimated by Kingsbury ('41) to contain at the time of his survey, more than 2,000 titles. The histologic changes which take place in this organ under various conditions, have been well illustrated and described in the extensive reports of Hammar ('21, '38), Gudernatsch ('37) and Nelson ('39), to mention but a few. Perhaps no feature of thymic alteration has been more intensively studied than that associated with age and accidental involution.

Before considering histologic changes appearing in the thymus under experimental conditions, it might be well to define several of the terms used in later descriptions. This is done because some of them have been variously used in the literature, often in at least partially contradictory form.

Without intending any commitment as to endocrine function, the thymus is referred to here as a *gland*. The term *stroma* designates the framework of the organ; this framework consists of fibers and fibrils, together with their associated cells. The stroma is most densely distributed in the lobular peripheries, and in the vicinity of the intralobular blood vessels. As far as is now known, the thymic stroma is of mesodermal origin (cf. Tschassownikow, '26; Popoff, '26, '27).

The term *epithelium* is used to denote the branching cords of thymic epithelial cells. This characteristic and constantly present element of the thymus forms a portion of the non-fibrous framework, and probably also supplies the cellular components of Hassall's corpuscles as well. Although stroma and epithelium cannot be differentiated in the normal gland under all conditions, they become easily distinguishable during experimental alteration of the organ. One such condition, that in which the thymus undergoes involution and regeneration, is considered in considerable detail in this paper.

Parenchyma is used to designate the collective thymocyte population of the gland. With few exceptions, this population consists of small thymocytes which are morphologically identical with small lymphocytes, but occasional thymocytes of medium and large size are encountered under normal conditions.

The use of the terms thymocyte and lymphocyte might be explained briefly. As noted above, these cells are morphologically identical, but there is still some doubt as to whether they have common origin and potentialities. This same incompleteness of knowledge exists for many elements of the body and of itself, should not merit coining new terms for description of the cells concerned. Notwithstanding, follow-

ing common usage, *thymocyte* shall be used here to describe the parenchymal element of the thymus, and *lymphocyte*, the morphologically identical cell in any other location. Comment will be made later, concerning identity of these cells under conditions of the experiments to be reported in this paper.

It has been found that, following induced involution, each thymic lobule is surrounded by a dense sheath which is primarily fibrillar in nature. This sheath or *capsule*, lies immediately peripheral to the surface of the lobule, and is itself embedded in the loose interlobular and perilobular connective tissue; the latter is a direct continuation of tissue from the superior mediastinum. The term capsule, as used in this paper, then, refers to the dense sheath of the individual lobule, and does not imply any continuous covering for the lobe as a unit or for the gland as a whole.

In following the concept of Hammar, many investigators have designated experimentally induced thymic degeneration as "accidental involution." Throughout the experiments to be described, the involution was in no sense accidental, and is accordingly termed *induced involution*.

Despite extensive investigation, workers have reached no general agreement on the sequence of events in thymic involution and regeneration, nor on the significance of the observed changes. Some of the questions still largely unanswered are the following:

1. Identity of the thymocyte. There are two principal schools of opinion as to the identity of the thymocyte. One group of workers has concluded that the thymocyte and lymphocyte are identical cells, and that they appear in the thymus only after invasion from without; these cells, then, would be of mesodermal origin. Among the proponents of this concept are Hammar ('09, '21), Jonson ('09), Maximow ('09, '12a, '12b), Pappenheimer ('10, '13), Rudberg ('07) and Wassen ('15). Gregoire ('43) has modified this concept somewhat by reporting that thymocytes are derived also from the perivascular connective tissue within the gland

substance. The authors cited above have minimized the importance of the epithelium in thymic activities. It is their opinion that although this tissue is consistently present in the gland, it plays little or no part in growth or in reconstruction and repair of the parenchyma. Primarily from the findings of Hammar in his extensive studies on involution, and from those of Maximow in a multitude of tissue-culture studies, the thymus is now rather generally considered as a *lympho-epithelial* organ.

The second group of investigators has insisted that the morphologic likeness of thymocyte and lymphocyte does not establish a common identity. They hold that the thymocyte is a derivative of the thymic epithelium and is thus of entodermal origin. They believe that this cell is formed *in situ* from the thymic epithelium. It can be thus seen that in particulars of both origin and mode of formation, according to this concept, lymphocytes and thymocytes would differ strikingly. The basic investigations which led to formation of this second school of opinion were carried out by Maurer (1885-86) and Stöhr ('06). Their findings were revised and extended by Gottesman and Jaffe ('26).

2. Fate of the thymocyte during involution. During the process of involution, it has been repeatedly observed that thymocytes disappear rapidly and almost completely from the organ. Some investigators (Deansley, '28; Gregoire, '43; Hammar, '21) have found that this is accomplished primarily through phagocytosis, and others hold that the elimination is brought about by cytolysis.

3. Components of thymic structure. Following the findings of Maximow ('09, '12b) and others, it is now accepted that as entodermal thymic epithelium develops in the embryo, it advances into the mesenchyme of the neck and, by a process of differential growth, is drawn downward into the superior mediastinum. Particularly in the earlier phases of this developmental procedure, the epithelial plates are broken up by ingrowth of mesenchyme which forms the interlobular connective tissue. The lobules are invaded by growing tips

of blood vessels and these become especially prominent in the medullary regions of the gland.

Special attention has been given to the composition of the thymic framework by Tschassownikow ('26) and Popoff ('26, '27). These authors contend that this framework is actually double in nature, one element being formed by a fibrous stroma of mesodermal origin, and the other, a much compressed epithelium, the remnant of the original entoderm of the pharyngeal pouch. In contrast to these observations, Deansley ('28) has concluded that the thymus is entirely devoid of an epithelial component.

4. Thymic phagocytes. Study of the available literature in this field has revealed no extensive investigation on the origin and reactions of the thymic phagocytes.

In the report which is to follow, each of the above problems is considered in some detail. This was done through the use of acid colloidal substances which, in small doses are but slightly toxic, and in larger doses definitely poisonous to the animals. These materials, in graded quantities, were injected into inbred animals of the same age. Under these controlled conditions, despite some degree of individual variation, any stage of the involution or regeneration process can be reproduced at will.

It will be profitable to review briefly the morphology of the thymus in the rat, with special attention to the features which are concerned in involution and regeneration.

The thymus is easily identified by means of its gross relationships in the superior mediastinum. The gland is normally grayish in color, and is composed of two lobes which are asymmetrically placed. Caudally, these lobes are approximated to the rostral surface of the pericardium and cranially, they extend into the root of the neck. The lobes are fused together by a sparse connective tissue net, but this connection is easily broken by gentle manipulation (cf. Green, '35).

The size, color and general relationships of the gland, and particularly its histologic composition, vary greatly at different periods in the life of the animal (Selye, '36; Smith,

'46). The rats used in these experiments were all 5-6 months of age at the time of their first injection. Control thymuses from this age group exhibited no individual variation which could be detected grossly, while differences in the histologic picture were too minute and inconstant to be of significance.

Each thymic lobe is composed of lobules which can be seen without magnification. The interlobular connective tissue is continued peripherally into the loose connective tissue of the superior mediastinum and root of the neck. The interlobular substance is structurally identical with that of the mediastinum except that the former typically contains a richer vascular plexus from which numerous small branches pass into the substance of the gland proper. It would thus appear that the thymus has a double vascular supply: one part is composed mainly of small arteries which gain ingress through the multiple hiluses of each lobe, and the other, made up mostly of vessels of arteriolar size, enters the lobules from many points on their peripheries.

In serial sections, it can be determined that the thymic lobe is formed of several principal stalks of tissue. Each stalk is profusely branched and there are several orders of subbranchings as well; the terminal divisions are rather bulbous. These terminal branches make up most of the lobular surface. At or near the central axis of the medulla, one or more small arteries are encountered, and these divide to follow the lobular branchings. Smaller divisions of the central blood vessels pass peripherally through each lobule and anastomose with small blood vessels which have reached the cortex by traversing the interlobular tissue.

All the intrathymic blood vessels are surrounded by a bed of perivascular connective tissue. This tissue is continuous with the adventitia of vessels in the superior mediastinum. Within the lobule proper, this tissue retains all its original structural characteristics. As will be brought out later, phagocytes derived from the intrathymic perivascular connective tissue, even in induced involution, are identical with

those occurring in loose connective tissue elsewhere in the body.

The general histologic characteristics of the thymus have been extensively studied and described (e.g., Hammar, '21), and need not be repeated here except for two special details. It will be remembered that in the rat, Hassall's corpuscles are small and inconspicuous, and that they rarely assume the typically laminated configuration characteristic of other animals. The second point deals with the zone of transition between the cortex and its immediately surrounding interlobular connective tissue. In the region of this transition, the loose, primarily collagenous interlobular framework undergoes a rather abrupt transformation; it is replaced at the border of the lobule by fibers of smaller caliber, and by a network composed of reticular fibrils. As far as can be determined, the lobular and interlobular connective tissues are directly continuous with each other.

The most conspicuous histologic component of the thymus, the thymocyte, is morphologically identical with the small lymphocyte. So numerous are thymocytes under normal conditions that the framework of the organ is largely obscured. Examination under favorable conditions (i.e., in regions where thymocytes are less closely packed) indicates that the normal thymus is supported by a stroma of collagenous fibers and reticular fibrils. These strands are associated with variable numbers of fibroblasts. Mingled with the stromal elements, are cords of epithelial cells; these cords are so greatly attenuated that they cannot be readily located in every region where the framework is available for study.

The thymic epithelial cells cannot be differentiated with certainty in the normal gland. The tissue usually appears as flattened masses in which the individual cells are stellate in form. The nuclei stain lightly after routine technical procedures, and contain only a few strands of chromatin material. The nuclear membrane is thin but sharply defined even under ordinary conditions.

As can be seen from the preceding description, epithelial cells of the normal thymus cannot be readily differentiated from fibroblasts or from the histiocytes of perivascular (mesodermal) connective tissue. This condition becomes quite altered after experimental involution, however, when the two cell types can be readily differentiated. In this instance, the epithelial cells undergo marked hypertrophy. It is believed that either the loss of thymocytes provides a strong stimulus for their increase in size, or that the process of destruction relieves them from simple mechanical compression. In either case, the experimental procedure makes accurate differentiation and study possible.

MATERIALS AND METHODS

The albino rats used in these experiments were of a single inbred strain. Whenever possible, littermates were used to make up the individual experimental groups. All animals were 5-6 months old at the time of the first injection. This age group was chosen because it provides young adult animals whose thymuses are of relatively constant histologic pattern.

Ninety-six rats were used in the following experimental groups. These animals were injected intraperitoneally with Chlorazol black E (National Aniline Co.) or with colloidal mercuric sulfide (Hille Laboratories) in the following manner:

- I. Acute series: 60 animals injected with Chlorazol black E or with HgS; of these, 19 were discarded and the remaining 41 autopsied and prepared for microscopic examination.

Group A: sustained acute dosage. Twelve animals were injected (6 for each of the two colloids) daily with 2 cm³ of 2% solution until death seemed imminent. Of this number, 5 died during the night and were discarded (three of the HgS series and two of the Chlorazol series). Two survived three doses and were killed on the 4th day of the experiment, while 5 survived two doses and were killed on the 3rd day of the experiment.

Group B: single injection without recovery period. Twenty-four animals (12 for each of the two colloids) were given

one injection of 2 cm³ of 2% solution, and the survivors killed at intervals between 12-36 hours after the injection. Of the original number in this group, 19 (12 of the Chlorazol series and 7 of the HgS series) survived more than the minimum 12-hour period, while 5 died during the night and were discarded.

Group C: single injection with recovery period. Twenty-four animals (12 for each of the two solutions) were given one injection of 2 cm³ of 2% colloid and the survivors killed at daily intervals beginning with the third post-injection day. Of the original number, 15 (10 of the Chlorazol series and 5 of the HgS series) lived through the term of the experiment, while 9 died and were discarded. The last member of this series was killed on the 17th post-injection day.

II. Chronic series: 36 animals were injected daily (6 times per week) with doses of Chorazol or HgS, regulated to subtoxic levels (cf. Baillif, '48). They were maintained under this regime until death seemed imminent, or until sacrificed to provide material for a series of glands representing weekly intervals. Some of the animals in this series (two injected with Chorazol) survived 7 months, at which time the experiment was terminated.

Some of the animals in the chronic series developed acutely toxic symptoms, at which time they were sacrificed immediately and prepared for microscopic study. These animals obviously must be assigned to the acute experimental series. Of the original number, 4 died during the night and were discarded, while 9 were killed while *in extremis*; thus, 23 thymuses of the chronic series were prepared for study.

After completion of the experimental period, the animals were killed with chloroform and autopsied immediately. Their thymuses were removed, fixed in Bouin, Helly or Zenker. Dehydration was begun in alcohol and completed in dioxane over calcium chloride. Serial sections were cut at 5 μ and stained with Harris' hematoxylin-eosin, or with Mallory's triple connective tissue stain preceded by hematoxylin. It was found that the finished sections were made materially clearer by allowing them to stand for several hours or overnight in xylol before mounting in clarite and covering.

OBSERVATIONS AND DISCUSSION

As explained in the preceding section, an attempt was made to maintain one group of experimental animals (II, the chronic series) with a maximum subtoxic dosage. Response under this regime was subject to considerable individual variability, even among littermates of the inbred strain. Certain animals remained in apparent good health and continued to gain weight (although at a reduced rate) for periods up to 7 months; others passed rather suddenly into an acutely toxic state. No explanation can be given for the variability of response, but it appears to be correlated with the degree of reticulo-endothelial reaction. In general, those animals with reticulo-endothelial tissues most heavily loaded with the injected colloid, appeared to suffer least and latest. This is particularly evidenced histologically by the presence of a dark layer composed of innumerable, heavily loaded macrophages which was found to occupy the peritoneal and subperitoneal loose connective tissues. After several months of repeated injections, certain regions of this tissue became jet black. Histologic sections reveal that all the colloid is here collected within macrophages; fibroblasts are never involved in process. Less resistant animals under identical conditions typically exhibited smaller concentrations of less heavily loaded macrophages.

On the basis of the above observation, it seems probable that various animals differ considerably with respect to their segregation potentialities. More resistant animals appear to be able to collect and activate macrophages in sufficient numbers to remove toxic quantities of the injected colloid. Less resistant animals, in contrast, lack the ability to form new macrophages in great numbers over an extended period, and thus the tissues are subjected to toxic effects of the colloid.

The early histologic changes which appear after injections of acid colloidal substances may be profitably discussed under two headings: acute and subacute involution. It is believed that these two processes are identical in every respect except

that of degree, but that this difference is of sufficient magnitude to make the separate discussions advisable.

Acute thymic involution

The principal findings in this set of experiments are summarized in table 1. Various points concerning involution are brought out in this table and discussed in the text immediately following it. As will be seen, the first and most conspicuous phase in the destructive process is the disintegration and removal of small thymocytes. Almost constantly expressed, and progressing as the small thymocytes are removed, is hypertrophy of the epithelial component. For reasons mentioned in the section on thymic regeneration, the epithelial reaction is considered to be regenerative, and will be treated in greater detail later in the paper.

The involuted thymus is smaller than the normal gland. As will be indicated shortly, the primary cause for this decrease is the profuse destruction and removal of thymocytes (figs. 1, 2). The superior mediastinum of these animals was flecked with collections of the foreign colloid; the normally pink color of the mediastinal tissue was replaced by gray or brown, depending on the degree of colloid collection. Due to this alteration in shade, the gross contrast between thymic lobules and their intervening connective tissue was considerably reduced. In extreme cases, it was not possible to locate accurately the extent of the thymus at the time of autopsy.

Interlobular connective tissue. The interlobular connective tissue of the involuted thymus appeared to have become looser in texture; it contained variable numbers of phagocytes within which were many globules of the injected material. At the surface of the thymic lobules, there was a network of condensed interlobular connective tissue. Within the interstices of this network phagocytes usually occurred in great numbers (fig. 3).

The capsule. As far as can be determined in preserved and sectioned material, the normal thymus is not surrounded by

TABLE 1
Thymic changes during acute involution and regeneration

| PERIOD (HRS.) AFTER APPEARANCE OF TOXIC SIGNS | GROSS APPEAR- ANCE OF THYMUS | MICROSCOPIC CHANGES IN THE LOBULE: | | PLASMOCYTES |
|--|---------------------------------|---|--|---|
| | | Cortex | Medulla | |
| 12-18 | No change. | Beginning hypertrophy (?) | Beginning hypertrophy (?) | Few in interlobular septa. |
| 18-24 | Minimal shrinkage. | Nuclear pyknosis in thymocytes; stromal hypertrophy. | Nuclear pyknosis in majority of thymocytes. | Many in interlobular conn. tissue; few at cortico-medullary junction. |
| 24-36 | Marked shrinkage. | Karyorrhexis and karyolysis; stromal hypertrophy. | Karyorrhexis and karyolysis in the majority of thymocytes. | Numerous throughout cortex; few in medulla; some degenerating. |
| 36-48 | Maximum shrinkage. | Few thymocytes remaining; stroma has open network. | Thymocytes few; stroma in hypertrophy. | Most numerous. |
| 48-72 | Marked shrinkage. | Few thymocytes; stroma in maximum hypertrophy; epithelial cells common. | Thymocytes common; stroma in maximum hypertrophy; epithelial cells in cords. | Rare in medulla; most common in outer cortex. |
| 72-96 | Shrinkage less marked. | Large thymocytes common in inner cortex. | Thymocytes numerous and in groups. Epithelial cords breaking into strands. | Mostly in outer cortex. |

a distinct capsule. In the involuted gland, however, it is covered by a thick, loosely matted sheath, composed of both fibers and fibrils, which is typically permeated by a network of small blood vessels (fig. 4). Within the meshes of the covering are found variable numbers of fibroblasts. The capsule merges insensibly with the loose connective tissue of the superior mediastinum and with that which fills the interlobular clefts. Adjacent to the surfaces of the individual lobules, the capsule becomes denser and is formed chiefly of fine fibrils. Especially in the fully involuted glands, these fibrils are observed to be continuous with the intralobular stroma. The meshes of the capsule contain several types of free cells, considered in some detail below. It has been difficult to determine precisely the origin of this capsule, but it is probably formed by condensation of the peripheral lobular stroma and of the adjacent connective tissue of the superior mediastinum.

The types and numbers of cells found in the capsule vary from gland to gland, and with the degree and stage of involution. Invariably it contains at least a few small thymocytes (cells identical morphologically with small lymphocytes), and there are usually scattered thymocytes (or lymphocytes) of medium size as well. Observations on all available instances of capsular infiltration indicate that at least most of these cells have migrated there from the thymic parenchyma. There is no indication of lymphocyte invasion from tissues which surround the thymus. During this migration, the small thymocytes are gradually transformed into those of medium size. While in the degenerative phase of thymic response, the number of thymocytes within the capsule is typically small, but in reconstruction they appear locally in considerable numbers. During that period, they may far outnumber the other cellular elements.

Early in the reconstructive process, the capsule frequently contains small groups of plasma cells. Although these elements show no cytological signs of deterioration, they have never been observed in mitosis. There are many indications

that small thymocytes within the capsule transform directly into plasma cells. Peculiarly enough, this process has not been noted within the meshes of the lobular stroma; it occurs only when the cells are relatively isolated and in an environment of rather dense connective tissue.

The transformation of thymocytes into plasma cells appears to be identical with that which has been described in formation of plasma cells from lymphocytes elsewhere in the body. In this process, a lightly staining, indefinitely marked zone (sometimes called the "Hof") appears in the center of the medium-sized thymocyte. This area enlarges and decreases in stainability as the nucleus recedes toward the cell periphery, and the cytoplasm not involved in the area becomes more basophilic. Throughout these alterations, the internal pattern of the nucleus remains unchanged. It is believed that the migratory activity of plasma cells in the thymus is rather limited, since few are encountered in the interlobular connective tissue under these experimental conditions. Cells of the type just described appear to migrate as thymocytes and to transform into plasma cells, mostly within the substance of the capsule.

Numbers of cells with acidophilic granulated cytoplasm can be found in the capsule, and to a lesser extent, in the interlobular connective tissue (figs. 2, 3, 8). Previously studied by Pappenheimer ('10) and others, these cells have been variously identified as atypically staining tissue mast cells and eosinophil myelocytes, as well as a number of other forms.

In animals of this experimental series, these granulated cells appear to have formed from small thymocytes for, even when fully laden with granules, their nuclei still remain lymphocytic in character (fig. 8). The tinctorial reaction of the cytoplasmic granules after Mallory's triple stain, for example, varies considerably with the type of fixative used. After Bouin, they are distinctly eosinophilic, but after use of Zenker or Helly they may be basophilic on occasion, or even metachromatic. This variability might be compared with

similar color differences noted in mast cells by Michels ('38) under the same technical conditions. On the basis of their morphologic and tinctorial similarities, it seems probable that the granulated cells of the thymus are but a variety of the tissue mast cell.

After repeated injection of foreign colloid, it has been found that these granulated cells take up the injected material. After use of either of the black colloids in quantity, the more peripherally placed granules in each cell lose their reddish color and may become tan, green, or even black after the same staining and fixation procedures. Since these tinctorially altered granules are otherwise identical with the unaffected, more centrally placed bodies, it is assumed that the former have adsorbed the foreign colloid. The granulated cells show no signs of phagocytic activity.

Pappenheimer ('10) has concluded that the granulated cells have a secretory function since they can be found in all stages of degranulation as well as with granule-free cytoplasm. These observations were confirmed in thymuses of both normal and experimental rats of this series (fig. 5), but it is believed that further evidence is necessary to establish these cytologic changes as a sign of secretory activity. It is considered more probable that the variable appearances represent stages in granule formation or differences of purely technical origin.

With the exception of fixed elements in the capsules of acutely degenerating thymuses, the most consistently appearing cells are phagocytes (fig. 9). This type of phagocyte may be either of stellate or of nearly spherical form; in the first instance its processes are retained even when the cytoplasm is heavily loaded with foreign colloid. It contains a central nucleus whose chromatin pattern is identical with that of macrophages found in the thymic interlobular connective tissue and in similar tissues throughout the body.

Phagocytes of ordinary connective tissue type are capable of ingesting quantities of foreign colloid. Globules of the material first collect in the perinuclear zone, but with in-

crease of number, they accumulate in the cytoplasmic processes as well (fig. 10). After distention of these cells, some of the loaded processes break free from the parent mass. Such isolated fragments can be identified in serial sections; they typically contain globules of the injected material, but these globules are in the process of disintegration, as indicated by a tendency of the entire fragment to stain diffusely; this reaction is characteristic of dead or dying protoplasm.

The ordinary connective tissue phagocytes appear to be almost incapable of carrying out digestion (decolorization) of the ingested colloid material. This is indicated by the observation that even when globules accumulate in great numbers, they still retain their intensely black shade (figs. 3, 9, 10). In isolated instances, there is a suggestion of fading at the center of the complex; in such cases, the black fades to brown (when the globules are of HgS) or to blue or gray when the globules are of Chlorazol black E. These features will be discussed again in the consideration of intralobular phagocytes; at that time, various contrasts will be drawn between connective tissue (or ordinary) and intralobular macrophages.

The cortex. After induced involution, the capsule becomes well defined and sharply demarcated from the underlying cortex. The transition between these two regions is morphologically expressed by abrupt appearance of the rather open pattern of cortical stroma (fig. 4). The framework in the cortex is formed by a sparse fibro-fibrillar network (the true stroma) and by the branched and anastomosing cords of epithelial cells. In normal thymus, the presence of innumerable thymocytes makes identification of these two components difficult. In the involuted thymus, this difficulty is largely removed; under these conditions, stromal fibers are but little altered, while epithelial cells increase greatly in volume. Despite increase in volume of the epithelial elements much of the space formerly filled with thymocytes remains empty in the involuting gland. This factor makes it possible to study the remaining tissues without interfer-

ence, although they are admittedly seen under abnormal conditions. This observation has been made repeatedly by previous workers (Dunn, '45; Foulds, '34; Goldner, '25; Gottesman and Jaffe, '26; Pappenheimer, '13; and many others). Deansley ('28), on the other hand, has been unable to find a persistent epithelial component in the thymus of mice, after treatment with x-ray.

The thymic epithelial cells are oval or stellate in form, and, in the involuted gland, distributed in cords which form an open network. The nuclear pattern is characteristic, the chromatin being collected into thin strands and granules which are most frequently gathered against the nuclear membrane. A prominent, central nucleolus is usually present, and there is a relatively great volume of faintly acidophilic nuclear sap. The large, pale nuclei of epithelial cells are thus easily differentiated from the smaller, densely basophilic nuclei of thymocytes (figs. 11, 12). Since both small and medium-sized thymocytes have their chromatin substance formed into block-like collections, its pattern is also useful in differentiating epithelial and thymocyte cell types. Attention is drawn to these peculiarities since they will be used later in differentiating the derivatives of the two cell types as well.

The cytoplasm of thymic epithelial cells is faintly acidophilic. It sometimes contains thin fibrils which appear to be branched. The number of these fibrils increases at the periphery of the cell, where they are merged with the cell membrane. The cell membrane is thin and but faintly chromatophilic; for this reason, and because they are closely packed in the cords, it is often difficult to delimit individual cells.

There is still a possibility that the thymic epithelium might be in part syncytial. Particularly by use of Mallory's triple connective tissue stain, however, it can be determined that the great majority of these cells are individual units. The only possible exception to this rule is in those elements which are centrally placed within the epithelial cord, though

in the best preparations the cells even here appear to be completely separated from each other (figs. 13, 14, 15).

Depending somewhat on the acuteness of the involution process and on the ability of each animal to produce these cells, all experimentally involuted thymic lobules contain colloid-filled macrophages. On the basis of position, they may be called intralobular phagocytes (figs. 4, 10, 17, 18, 19). When there has been an abundant supply of foreign colloid in the tissue spaces over an extended period, these cells become numerous; they are typically large and spherical. Even when loaded with ingested material, their nuclei remain centrally placed. The chromatin pattern of these nuclei is identical with that described above for thymic epithelial cells; i.e., it is composed of strands and granules chiefly condensed beneath the nuclear membrane.

Intralobular phagocytes contain colloid globules in varying size and number. These globules are largely unaffected by the procedures of histologic preparation; they range in color from black to brown or tan after injections of HgS, or black to blue or even pink after Chlorazol black E. In either case, lightening of the shade probably expresses disintegration or digestion of the globule. In no instance have these inclusions been completely decolorized; it is thus assumed that the digestion process cannot be carried to complete solution, but is arrested when the colloid has been reduced to a lightly colored, homogeneous mass. Frequently these masses are of considerable volume, and are probably formed through coalescence of many of the smaller, formerly black globules.

In cases where intralobular macrophages are large and numerous, they are frequently collected into groups. On progressive increase in size, the individual elements of such a group become compressed against the surrounding framework of the cortex and into intimate contact with each other. This seems to predispose toward fusion, and it is not unusual to encounter giant cells in this location containing as

many as 7 or 8 nuclei. Even under such conditions, the nuclei retain the epithelial-cell characteristics (figs. 19, 20).

It should be noted again that the phagocytes in the lobular capsule and interlobular connective tissue differ considerably from those encountered within the substance of the lobule proper. These differences may be expressed in tabular form as follows:

TABLE 2
*Differences between macrophages of connective tissue
and intralobular types*

| | ORDINARY CONNECTIVE- TISSUE MACROPHAGES | INTRALOBULAR MACROPHAGES |
|---|--|---|
| Distribution | Isolated, colloid-filled; found throughout loose connective tissue and in thymic interlobular connective tissue (capsule). | Cells appear singly or in groups within the thymic lobules. |
| Cell form | Oval or stellate; isolated, colloid-filled cytoplasmic tongues may break from the parent cells. | Nearly spherical to spherical; giant cells appear after repeated injections. |
| Nucleus | Flattened or oval; internal pattern of the typical tissue histiocyte. | Spherical; internal pattern like that of the thymic epithelial cell. This pattern retained after cell distortion. |
| Phagocytosed inclusions | Numerous. | Less numerous. |
| Coalescence of ingested globules | Delayed; inclusions often remain small and discrete even when numerous. | Early and prominent. Complexes formed may exceed nucleus in diameter. |
| Digestion (decolorization) of ingested globules | Feeble or absent. | Prominent; larger inclusion complexes usually pale in color; may be acidophilic. |

Even during the most severe involution, the cortical stromal spaces invariably contain a few small thymocytes which show no signs of degeneration. It is believed that these elements have escaped the destructive process and will later assist in reestablishment of the multitude of thymocytes which appears during reconstruction. Throughout involution and early regeneration, however, only a few mitotic figures can be seen in the persisting thymocytes. For this reason, thymocytes formed during early reconstruction must be derived largely from some other source which, as will be shown later, is the epithelial component of the regenerating thymus.

The medulla. The medulla of the involuted thymic lobule is not sharply demarcated from the cortex. After involutionary loss of small thymocytes, the medulla also contains many open spaces in whose boundaries both stromal and epithelial components can be distinguished. As in the normal thymic lobule, the involuted medulla is supported by a fibro-fibrillar stroma and by an epithelial reticulum; the meshes of this spongy framework contain many blood vessels. Hassall's corpuscles persist in relatively unaltered form, but since the rat thymus normally contains but a few of these bodies, they never constitute a conspicuous element of the involuted medulla.

The types of free cells mentioned in the preceding section are encountered also in the involuted medulla. Acidophilic cells occur but rarely, phagocytes appear in varying numbers depending on the degree of involution, and the free thymocytes are gathered in small groups. The phagocytes in this region are of both the previously described types. A few, found in the perivascular connective tissue, are of connective-tissue type; the majority appear in the interstices of the framework and are of intralobular type.

Toxic reaction in thymic cells. As is now well known, thymic involution is invariably characterized by profuse degeneration and rapid removal of thymocytes (figs. 1, 2). In most instances, this change can be detected by the appearance of pycnotic nuclei as early as 18-24 hours after the injection

of the acid colloid in toxic dose. The process of thymocyte destruction is largely completed within a 48-hour period in cases where the maximum tolerated dosage was used.

The destruction of thymocytes during induced involution is carried out in a series of stages: pycnosis, karyorrhexis, and finally, complete cytolysis. The phagocytosis observed under similar conditions by Deansley ('28), Gregoire ('43), and Hammar ('21) did not take place to any notable degree in involuting thymuses in this experimental series.

Phagocytes loaded with foreign colloid are commonly seen under these conditions. As previously stated, this segregated material cannot be completely digested, but remains as pale, acidophilic masses within the cells. By contrast, ingestion of dead or dying cells such as thymocytes may be followed by such rapid digestion that the intracellular debris does not collect in any significant volume. It has been noted occasionally that intralobular phagocytes sometimes contain isolated, colorless vacuoles mingled with the colloid complexes; these colorless vacuoles may represent the terminal phase of thymocyte digestion (fig. 16).

Not all parts of the thymus undergo involutional changes at the same time; for this reason, the true picture of time lapse and thymocyte destruction can be gained only after examination of the entire organ. Indeed, there can be considerable variation in the histological condition, even within parts of the same lobule. One explanation for this phenomenon is that there is great difference in the susceptibility of various thymocytes to toxic action; this variation may be due to differences in cell lineage. On examination of thymocytes of various sizes, it was found that in this type of induced involution at least, the toxic susceptibilities are expressed in the following relation to cell size: small thymocytes > medium thymocytes > large thymocytes.

The toxic reactions of large thymocytes still remain in some doubt due to the paucity in their numbers. From study of available examples of large thymocytes, however, it appears that they have retained all the potentialities of the two

smaller thymocyte types. These cells remain viable after toxic doses of the injected material; they also retain their ability to reproduce and to transform into related cell forms.

As has been indicated above, the thymic lobule at the height of involution typically exhibits many open spaces in which lies the debris of degenerating thymocytes. In addition, three of the animals in this experiment presented, even at this period, numerous segregating phagocytes. It is assumed that these animals had reticulo-endothelial systems which were more quickly stimulated to phagocytosis than was the usual case.

A few disintegrating intralobular phagocytes occurred in all involuting glands, but many were found in the three animals mentioned above. Frequently, the sites of degeneration within these lobules were marked by newly freed collections of colloid globules mixed with the cellular debris. It was noted further, that phagocytes of ordinary connective tissue type were not affected in the same manner as those just described. These cells were never found in a state of degeneration even in thymuses of the most toxic animals. They appear to be functionally still active and adding to their ingested store, in animals sacrificed *in extremis*.

Subacute involution

Not all thymuses of the experimental animals react in the manner described in the preceding section. When dosage has been maintained at subtoxic level (series II), the animals appear to suffer but little physical harm. The colloidal materials used are, however, but slowly eliminated from the body, and thus accumulate progressively as the injection period is prolonged. For this reason, the toxic symptoms often appeared quite without regard to how greatly the dosage was decreased, toward termination of the experiment. Histologically, the thymuses of subtoxic animals showed the usual signs of involution, but these alterations were not as extensive as in the glands previously described.

The initial changes in thymuses undergoing subacute involution (i.e., glands from animals of series II sacrificed before they developed acutely toxic symptoms) were identical with those seen at the beginning of acute involution. There is destruction of small thymocytes and pronounced hypertrophy of the epithelium, the latter being progressively enhanced as thymocyte destruction progresses.

The periods at which these changes appear are so individually variable that only generalizations can be made. Pycnosis and karyorrhexis in thymocytes can be detected usually within 3-5 days after the initial dose. Within the first week of injections, ordinary connective tissue phagocytes appear in numbers and begin segregation of the injected material, but intralobular phagocytes are typically few and may be absent. When present only in small number, these latter cells are first found at the cortico-medullary junctions of the lobules.

Subacute involution appears to be transitory, since regenerative changes are frequently prominent before destruction is completed. Even more strikingly indicated, in these instances, is the histologic variability of different lobules within a single gland. Some may show signs of rather acute thymocyte destruction, others of minimal destruction, and still other lobules are frankly regenerative in character. The accurate evaluation of subacute thymic involution, then, is dependent on careful examination of all parts of the organ.

Regeneration

The process of regeneration has been repeatedly used in the past to determine the activities and potentialities of various thymic elements. They have been studied in tissue culture, for example, by a number of workers. Most investigators using this method (e.g., Emmart, '36; Jaffe, '26) have experienced no particular difficulty in identifying the cellular elements and in following their history under various conditions. It is the opinion of Tschassownikow ('26) that the

thymic framework is of dual nature, and that it produces two lines of functionally different cells. One of these behaves like typical epithelium in culture, and the other like reticular or resting wandering cells. The latter form is the source for thymic phagocytes and presumably, for new thymocytes. These findings support the lympho-epithelial concept of Hammar and of Maximow.

Both the above investigators were able to follow the thymic epithelium in their preparations, and Murray ('47) has devised a special technique for growing this tissue in pure culture. Under the dedifferentiating conditions of this method, however, it is still difficult to determine its full potentialities. Nonetheless, Murray has been able to find isolated instances in which it appeared that the epithelial cell, by atypical mitosis, formed one new thymocyte and one epithelial daughter cell. He did not find that phagocytes were produced in this process, although some of the cells were active in pinocytosis.

Almost all *in vitro* studies indicate that the cultural characteristics of thymocytes and lymphocytes are identical, not only in morphologic features but also in those functional characteristics which can be carefully controlled and followed. These findings still further weaken the hypothesis that thymocytes and lymphocytes represent separate cell lines.

Morphologic indications of thymic regeneration vary somewhat, depending on the method by which involution was induced. In general, however, the same features in varying degrees of prominence can be seen in animals from all experimental groups used in this investigation.

Epithelial hyperplasia. The earliest sign of thymic regeneration, epithelial hyperplasia, can be detected usually by the end of the first day after injections were begun. This is to be considered as a regenerative reaction since its degree is at least roughly proportional to the extent of thymocyte destruction, and since it culminates in formation of great numbers of new thymocytes. As indicated in the section on

acute thymic involution, epithelial cells and their responses are best studied in the involuted gland, before the thymocytes have been replaced in numbers.

As soon as about half the thymocytes have been destroyed, the epithelium becomes readily visible even though its hypertrophy is still minimal (figs. 11, 12). At this time, the cells can be identified in cords which are more prominent in the medullary regions of the lobules. In their maximum development the cords may be as many as 8-10 cells in thickness, but 3-5 cells is more common. They are richly branched, and serial sections indicate that these subdivisions closely follow the branchings of the stromal framework which makes up the lobule. Smaller branches of the epithelial cords project into the lobular cortices and there terminate in blunt expansions (fig. 13). Especially in these terminal regions, and to a lesser degree along the margins of the cords throughout their extent, there is an admixture of fixed (epithelial) and free or partially freed ("prethymocyte") cells (fig. 14). These relationships are discussed in more detail below.

With the advent of hyperplasia, the epithelial cells individually increase in volume, and their nuclei bulge into the open spaces of the lobular framework. Even when cells of these cords are stained with special techniques, it is often difficult to detect limiting membranes. Typically, these boundaries are more definitely and easily stained in cells which lie at or near the peripheries of the cell cords, but the more centrally placed elements are not surfaced by similarly chromatophilic membranes. If any of the thymic epithelial cords are syncytial, this condition is confined to the interior of the cord substance. As the cells are moved peripherally in the cords by removal of the surface elements, they acquire more definitely stainable cell membranes (figs. 14, 21, 22). The extensive fibrillar reticulum described by Douthat and Pardinas ('43) can be readily seen in these thymuses. In addition, its relationship to the hypertrophic epithelium can be determined with considerable accuracy. Under these conditions, it appears that the reticulum is associated primarily

with the stroma proper, and only secondarily with the epithelium.

Not all epithelial cells in the regenerating thymus undergo hypertrophy at the same time, or to the same degree. During hypertrophy, wide variations appear in the thickness of their cytoplasmic processes, and in the diameter of their nuclei. The nuclei become progressively less chromatophilic as they increase in volume. This process is associated with the redistribution of stainable material within the hypochromatophilic nuclear sap.

Animals subjected to toxic doses of the injected material (series I, group C) have thymuses with the most highly hypertrophied epithelial cells. By the close of the third day, epithelial cords in these thymuses have become the most conspicuous element of the organ. In such instances, their nuclei may reach 35μ in diameter.

Within the epithelial cords, individual cells are so orientated that their long axes are parallel with that of the cord as a whole. In some places, the cord is hollowed to form tubular structures previously termed "thymic follicles"; these are of irregular diameter and depth (figs. 13, 15, 23). The lining cells of such follicles have low columnar or cuboidal form and somewhat resemble the primitive lining of developing branchial pouches. Such bodies have been reported to occur under similar conditions of thymic regeneration by a number of authors, e.g., Selye ('36) and Smith ('46). The cytoplasm of epithelial cells in general, and of follicular cells in particular, is stippled with acidophilic granules. In some instances, the follicles are partially filled with an amorphous, acidophilic mass; it is possible that this mass represents collected secretion and that the granules mentioned above are secretion precursors.

In certain portions of the epithelial cords, the cells are spindle-shaped and of considerable length. The cytoplasm of such cells is similarly marked with an acidophilic deposit, but here it is formed into longitudinally directed fibrillar strands, somewhat resembling primitive myofibrils or the

tonofibrils of stratified squamous epithelium (cf. Dunn, '45; Foulds, '34).

During early hypertrophy, thymic stromal fibers are crowded aside by the enlarging epithelial cells. The surfaces of these epithelial cells are thus compressed against the displaced stromal network. In certain regions, cells at the surfaces of the epithelial cords begin to assume spherical form, and to free themselves, escaping into the interstices of the open stromal network. Where this process is active, rows of the newly freed, rounded elements adjoin the epithelial cords or are separated from their parent cords by the stromal fibers (fig. 14). This relationship is speedily lost, however, as is also the disposition of the newly freed cells into rows. Growth pressure, or possibly active migration forces these cells out into the stromal spaces in numbers, gradually refilling them. The relationships and cytology of the above cells are easily studied during the period in which the stromal spaces are being repopulated.

During this period of reorganization in the thymic lobule, careful search was made for signs of activity in fixed stromal cells. In exactly the same manner as these flattened, stellate elements remain unchanged during thymic involution, they are also quite unchanged in the regeneration process. In all instances, the morphology of these cells indicates that they are fixed fibroblasts; as such, they do not readily become active during thymocyto-genesis (figs. 4, 24).

Study has been made also of the intralobular perivascular connective tissue and the loose interlobular connective tissue; these were searched for signs of thymocyto-genesis and for signs of cell migration. The intralobular perivascular tissue remains almost unaltered under these conditions. Its fibroblasts show no signs of either hypertrophy or hyperplasia, while the perivascular histiocytes remain small, flattened and relatively inconspicuous. The occasional signs of histiocytic cytotransformation appear to be confined to production of a few new phagocytes of the ordinary connective tissue type. There were no collections of lymphocytes in

the interlobular connective tissues; indeed, it was often difficult to find even isolated lymphocytes in this region.

It is at present quite impossible to determine whether lymphocytes might be brought to the regenerating thymus *via* the blood vessels, and discharged there after passage through capillary or precapillary walls. Since thymocyte repopulation centers are not typically localized in relation to the intralobular blood vessels, it seems highly improbable that this process is of any great importance. The above observations are not in accord with findings of several of the earlier workers on thymic regeneration (e.g., Gregoire, '43; Rudberg, '07). It was the opinion of such investigators that repopulation of the involuted thymus is mainly if not exclusively effected through invasion of the gland by lymphocytes, and that these invading cells become the new thymocytes.

In the present study, it has been noted that reorganization in the involuted thymus is initiated by liberation of numerous cells from the hypertrophied epithelial cords. This is unaccompanied by thymocyto-genesis from activity of the mesodermal cells of the stroma, or of the intralobular perivascular connective tissue. There were no signs of lymphocyte invasion from the interlobular connective tissue.

Formation of thymocytes from epithelial cells. As the epithelial cells separate themselves from the parent cords, they undergo a series of pronounced morphologic changes which are particularly prominent in the nuclei. At the time of separation, these nuclei are large, and they have retained the typical epithelial-cell chromatin pattern. The newly freed elements contain nuclei which are smaller than those of the parent cords, and which appear to contain a greater quantity of stainable chromatin. This material becomes progressively concentrated into block-like masses which are distributed throughout the nucleus. While these nuclear changes are under way, the formerly almost chromophobic cytoplasm becomes more basophilic, and its periphery limited by a thin but sharply chromatophilic cell membrane (figs. 14, 15, 21,

22). With these cytologic alterations, the epithelial cell is transformed into an element morphologically identical with the medium lymphocyte; this element is here designated as "thymocyte" only because of its location in the thymus.

Despite the fact that many cells can be found freeing themselves from epithelial cords of the regenerating thymus, medium thymocytes never accumulate in numbers within the intralobular spaces. As they pass farther and farther from the parent cord, the great majority of these elements continue their transformation. They decrease in size and their nuclei become more densely chromatophilic, due to condensation of the contained chromatin into blocks which are not sharply demarcated from intervening parachromatin substance. The volume of the latter is greatly reduced in the transforming cell. As these alterations are effected, the medium thymocyte gradually assumes the characteristics of a typical small thymocyte. During the regeneration of parenchyma, then, the cell type which accumulates there is the small thymocyte. In animals of the toxic series, the primary source for these new cells is the entodermal, epithelial component of the gland.

Cells undergoing the changes described in the preceding paragraphs (from epithelial cell to medium thymocyte to small thymocyte) can be found in every regenerating gland. The decrease in size of cells during transformation, and the absence of mitoses in the repopulation mechanism, are unusual features of the process.

There is still some doubt as to the migratory ability of thymocytes in tissues. It appears that on arising from the epithelial cords, the majority of these cells pass into the stromal spaces and there complete their transformation. In isolated instances, certain medium thymocytes continue to migrate until they reach the lobular capsule; this more protracted migration becomes prominent only after the parenchyma has been largely regenerated. While in the capsule, they may continue transformation into small thymocytes, or they may become plasma cells. Should such small thymocytes

escape into the perilobular connective tissue as indicated in figures 6 and 7, they would obviously be indistinguishable from hematogenous lymphocytes.

As already noted, not all cells within the capsule are of thymocyte type, since a few have transformed into plasma cells. After careful search, all gradations between medium thymocytes and plasma cells were found here. Degenerating cells, either thymocytes or plasma cells, were not encountered. Plasma cells have been previously described in the rat thymus by Ross and Korenchevsky ('41), but these authors have found them principally in the interlobular connective tissues.

Thymocyte formation from preëxisting thymocytes. In every gland, some thymocytes escape destruction during the process of induced involution. This observation has been made repeatedly by other investigators, the majority of whom have found that the resistant thymocytes undergo early and active multiplication to repopulate the regenerating gland (Foulds, '34; Goldner, '25); Deansley ('28) has observed evidences of thymocyte multiplication by amitosis.

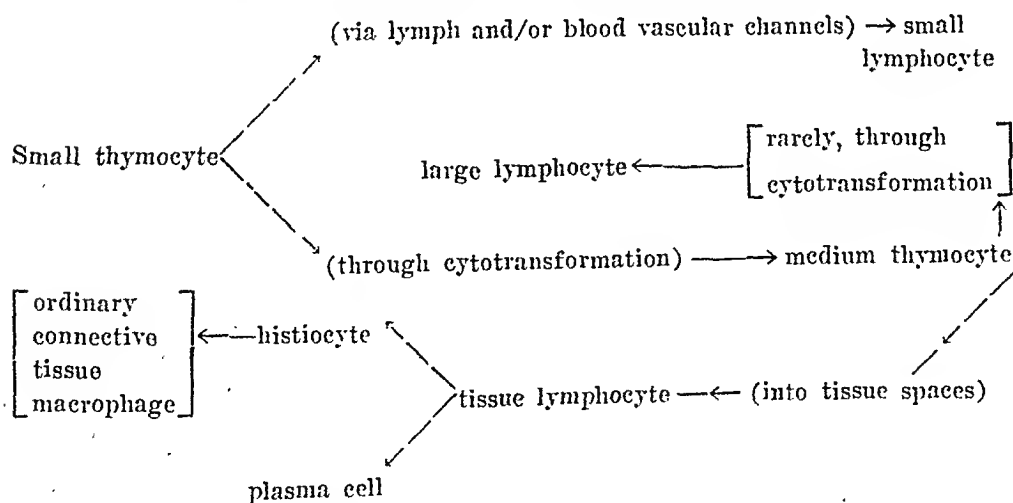
Peculiarly enough, mitotic figures are exceedingly rare during thymic regeneration after colloid induced involution. Study of serial sections of early regenerating glands has revealed that more than half of all the lobules are without mitotic figures, while the remainder show only a scattering of dividing cells. Signs of amitosis were lacking and there was no indication that lymphocytes were invading the regenerating gland. It appears, accordingly, that the initial replacement of thymocytes must be accomplished largely through transformation of epithelial cells.

Those thymocytes which escaped destruction may play a part in reorganization of the thymus, however, as is indicated by the presence of mitotic figures in many small thymocytes during later regeneration. The relatively late appearance of mitoses is noted here, reserving discussion of thymocyte relationships for a later section of this paper.

Kindred ('40) has carried out extensive studies on mitoses in the lymphocyte population of various lymphocytopoietic

organs in young albino rats. His findings indicate that the thymus produces more thymocytes than are needed for its own parenchymatous development. Since the thymus is an organ with rich lymphatic drainage (Matsunaga, '10), it would be quite possible that these excess cells could drain into circulatory channels of the body. Kindred has also calculated that the production rate of thymocytes in young rats "is equivalent to about 20% of the small lymphocyte population of the blood if the mitotic cycle of the thymic cells is reckoned as of one hour in length." Andreasen and Ottesen ('44) have reached similar conclusions by another experimental method. If it can be assumed that the small thymocytes are still produced in excess of need in the 5-8 month rat thymus, and that these excess cells become circulating lymphocytes, the present observations indicate that at least a portion of the blood lymphocytes are of entodermal origin. These and other relationships of the small thymocyte, based on observations made during studies on thymic involution and regeneration, can be expressed profitably in tabular form.

TABLE 3

Functional potentialities of the small thymocyte

Origin of phagocytes in the regenerating thymus. The thymus of every animal subjected to colloid injections shows at least some degree of epithelial hyperplasia. As previously

indicated, the more peripherally placed cells in the hyperplastic epithelial cords are prone to pull free and to escape into the stromal spaces of the gland. In some instances, almost all these freed cells undergo immediate transformation into thymocytes. As the dosage of colloid is increased and animals carry increasing concentrations of the foreign material, the process of epithelial thymocyto-genesis is progressively depressed. Under these conditions, a great proportion of the freed cells become phagocytes.

In the transformation from epithelial cell into phagocyte there is no decrease in cell size, and the nucleus never loses its original pattern. These large, pale cells begin to show morphologic signs of phagocytic activity almost as soon as they are freed (fig. 16). At first, their cytoplasm encloses but few globules of the foreign colloid, but when the available supply of this material is large, the intracellular accumulation increases rapidly and becomes densely black (fig. 17). The ingested particles undergo coalescence and early digestion. The latter process is not completed, however, in that the digesting cells remain loaded with masses that are only partially decolorized (figs. 4, 6, 10, 18, 19, 20, 25).

The differentiation of intralobular phagocytes from epithelial cells seems to be carried out in waves. This is indicated by the fact that phagocytic activity appears simultaneously in groups of the newly freed cells which lie near the margins of an epithelial cord. These cells retain their rounded form, but progressively increase in volume as the ingested material accumulates. While these changes are being effected, another generation of cells becomes freed from the cord surface, but these latter elements show no evidences of phagocytosis until the process has reached considerable magnitude in those previously freed. In toxically injected animals, where there is ample supply of the colloid material, giant cells may appear through coalescence of phagocytes before more than two or three waves of phagocyte formation have taken place.

Giant cells formed from intralobular macrophages are common in all thymuses of the acute series except those from animals given a single toxic injection without recovery period (group B). This probably means that giant cells only arise following a considerable accumulation of the injected material and compression of adjacent macrophages of the group; both processes are effected rapidly in highly toxic animals.

Giant cells formed from intralobular macrophages have from three to as many as 9 nuclei (figs. 19, 20). They invariably contain globules of the ingested colloid, collected into masses varying greatly in size. Some may exceed the diameter of one of the nuclei, while others are still at the lower limits of microscopic visibility. Digestion of the foreign colloid is indicated by progressive fading in its color, and is particularly noticeable in the larger conglomerates. In certain cases, the original color is completely lost and is replaced by one which is in some shade of yellow or pink after eosin staining. Many of the conglomerates are suspended in colorless vacuoles, but it has been impossible to determine whether this peripherally placed fluid is derived from complete digestion of part of the enclosed colloid, or from collection of cellular hyaloplasm. In either case, the cell seems to be quite unable to complete its entire dissolution. The giant cells which contain these digesting masses show no marked signs of cytologic disintegration.

Later phases of lobular regeneration. Regeneration in the thymic lobule can be completed as early as the 7th day after a single toxic injection of the colloid. A few animals in this experimental group developed acute initial toxicity, but the others suffered only temporary signs of illness. In the majority of the latter instances, histologic sections of the thymus show that many of the thymocytes have escaped destruction, and that they were able to take part in the regeneration process. Epithelial hypertrophy was invariably present, but less pronounced than in cases of more toxic and less precocious regeneration. Phagocytes of the intralobular

type were typically found only at the cortico-medullary junctions, or were entirely absent.

As early as the third day after a single toxic dose of the colloid substance, numerous small thymocytes of epithelial origin are present in both cortex and medulla of the regenerating lobule. By this time, such thymocytes cannot be distinguished morphologically from those which escaped destruction in the involution process. Apparently, the cells from both sources have now reached a state of functional maturity and are now prepared to undergo mitosis for replenishment of the thymic parenchyma. Numbers of these cells can be seen in mitosis at this phase of regeneration; typically, the mitotic figures are found in groups of contiguous cells, and most of these groups appear in the cortical regions. By the above process, there is soon established a marked preponderance of free cells in the more peripheral parts of the lobule.

As the regenerating lobule is refilled with thymocytes, the epithelial cells become progressively less prominent. The cords gradually disappear as such, being broken up into smaller strands by interposition of thymocytes, while individual epithelial cells are flattened and compressed during the process. In this manner, the epithelial cells are flattened against the lobular stroma, and soon become indistinguishable from the reticular and perivascular elements which comprise the stroma proper (fig. 26). The last region of the lobule to lose its hypertrophied epithelial component is the cortico-medullary junction. When epithelial reaction has been pronounced, this zone still may be marked by numerous, small, discontinuous strands of epithelial cells when all other parts of the lobule are fully reorganized. In cases exhibiting pronounced phagocytic activity, colloid-filled macrophages remain here after those in other parts of the lobule have been destroyed and their debris wholly removed. Eventually, however, phagocytes disappear from this zone also, and only macrophages of ordinary connective tissue type remain.

During the later phase of lobular reorganization, the capsule becomes thinned when its inner portion is refilled with thymocytes. By days 8-12 after a single toxic injection, not only has the inner portion of the capsule been incorporated into the cortex, but its more peripheral part has become less densely fibrous and identical with the interlobular connective tissue. There is no apparent structural difference between these two regions of the thymic capsule, but some mechanism appears to limit sharply the peripheral migration of the cortical thymocytes to form the outer border of the reorganizing cortex. In the manner described above, the capsule, a prominent structural feature of the involuted gland, disappears almost completely while regeneration is being accomplished.

Regeneration under highly toxic conditions. It will be remembered that one experimental group (group C, series I) was composed of animals which were given a single toxic dose of the colloid and allowed varying periods for recovery. It was found that three of these surviving animals (killed on post-injection days 7, 11, and 12) exhibited a more profound thymic response than did the others. These animals had thymuses which contained many more phagocytes of both intralobular and ordinary connective tissue types. So great was the quantity of fixed colloid in these animals that their thymuses could be identified grossly in the superior mediastinum on the basis of darker color alone.

All these highly toxic animals showed signs of thymic regeneration, but the process was not so advanced as that in less toxic animals of the same experimental group. In addition, the regenerative signs were of somewhat different character. It is assumed that these peculiar manifestations of regeneration were engendered by the presence of the injected colloid in considerable quantity.

The thymuses of these highly toxic animals were characterized histologically by the most extreme epithelial hyperplasia, and by great numbers of large, pale cells which had pulled free from the epithelial reticulum (fig. 25). These

freed cells, in contrast with those in less toxic glands, seemed to be chiefly transforming into intralobular macrophages. Cells undergoing thymocytopoiesis were infrequent and in some regions completely missing. Phagocytes found in these conditions were scattered throughout the lobular spaces, and many of these heavily loaded cells were in the process of giant cell formation.

Study of the intralobular thymocytes formed under these highly toxic conditions revealed that many were thrown into immediate pycnosis and subsequent degeneration; all glands were marked by degenerating thymocytes in regions of active regeneration. The greatest concentration of degenerating cells was encountered in the subcapsular region of the cortex.

Not only do intralobular phagocytes and small thymocytes undergo degeneration under these conditions, but a similar reaction takes place in isolated locations where thymocytes of medium size are to be found. These larger cells are most frequent in the cortex where they occur in groups. Such cells continue to increase in size until they may become the largest of the free structural elements, even exceeding the diameter of a single, loaded intralobular macrophage. In this growth, the thymocyte nuclei lose their typical block-like chromatin pattern; this material becomes gathered into attenuated bands which are only mildly basophilic. Concomitantly, the nuclei become polymorphic, being drawn out into two or more lobes of irregular outline; the margins of these lobes are marked by many spiny extensions. The cytoplasm of such cells is muddy in appearance, and the cell itself soon undergoes cytolysis.

In studying the susceptibilities of cells in thymuses from these highly toxic animals, it has been found that, although phagocytes are sometimes destroyed, they are typically more resistant than are the thymocytes. In no instance was there response in elements derived from the thymic stroma proper. The schema of toxic susceptibilities in cells (cf., p. 477) can now be extended as follows: small thymocytes > medium

thymocytes > large thymocytes > intralobular phagocytes > stromal histiocytes and reticular cells.

Thymic response in subtoxic animals. The animals of series II were maintained as long as possible by attempting to inject the colloid substance in subtoxic doses. It was possible to keep these animals in subtoxic state for varying periods of time by regulating the dosage with respect to the behavior and external appearance. With few exceptions, however, it seems probable that all animals eventually would have become toxic if sufficient time under this regime had been allowed. It is assumed that this terminal change is due to a progressive accumulation of the colloid until its disintegration products reach such concentration as to become toxic.

Thymuses of animals maintained at subtoxic levels showed signs of induced involution, but these signs never reached the degree of magnitude encountered in highly toxic glands. In all cases, thymuses from chronic animals exhibited diffuse pyenosis, at times involving most of the small thymocytes. The second phase of the reaction, that of karyorrhexis, was present only in isolated areas of the glands, and was sometimes found only in scattered cells. The third phase, that of karyolysis and cytolysis, was usually absent. This probably means that the destructive process was present but that it was slight in degree, and thus only incompletely indicated in histologic preparations.

The thymic epithelial cells in all chronically treated animals showed mild hypertrophy. The nuclei of these cells were enlarged and their containing cells often collected into groups, especially in the medulla of the gland; in no instance was there formation of the prominent epithelial cords described in the preceding section. It was difficult to trace all stages of separation of cells from this mildly hypertrophic epithelium, and also their transformation into thymocytes. Large, pale, nearly spherical cells with epithelial-type nuclei were frequently encountered in the cortical framework, but, by analogy, it is thought that they were derived from epithelium.

The thymuses of animals from this group appear to indicate the effects of chronic stimulation with regard to thymocytes, as well as to the other structural elements. This is evidenced by the fact that under these conditions, thymocytes, and particularly those of medium size, undergo rather extensive migration. Such migrations are most striking in animals maintained at subtoxic levels three months or longer. The migrating cells were found collected in groups in the capsular regions of the lobules; in mildly hypertrophic thymuses such as these, the capsule is not sharply demarcated from interlobular connective tissue. Occasionally, small numbers of medium thymocytes which migrated into the capsule were there transformed into plasma cells, but plasma cells were rarely encountered in any considerable number, either in the capsule or interlobular connective tissue.

As was the case in toxic animals, early thymic regeneration in subtoxic animals was not attended by extensive multiplication of small thymocytes. Careful search has been made for mitotic figures in all parts of these glands but they have been found in only isolated instances. From this observation it would appear that new thymocytes, in this instance also, are produced by cell transformation rather than by multiplication of preëxisting elements. Since a great proportion of the parenchyma escapes destruction in thymuses of subtoxic animals, however, the above process is probably not of great importance in regeneration.

Two animals of this series (one maintained for 6 months and the other for 7 months) had thymuses with minimal signs of involution; the glands were abnormal only with regard to the presence of hypertrophied epithelium and considerable numbers of migrating thymocytes. On the basis of the above observation, it seems possible that the chronically toxic processes, during the rather extended period of experimentation, had destroyed all the more susceptible thymocytes. These had been replaced by morphologically identical, but functionally more resistant cells. Hypertrophy of

the epithelium indicates that there was still some call on the replacement mechanism.

The thymuses of chronically injected animals invariably showed numbers of colloid-filled macrophages. These cells were of both the ordinary connective tissue and intralobular types. The connective tissue macrophages were more numerous than in any thymuses taken from animals of the acute series. This is probably due only to the length of time which the cells had to collect the particulate materials. Intralobular macrophages were always few, and occasionally difficult to locate at all. No giant cells were encountered.

The small degree of collection and isolation of the injected colloid material in chronically injected animals may be associated with a paucity in its available supply. It might be assumed that the foreign colloid was almost completely filtered from blood and tissue fluids before they reached the thymus. If this is true, mobilization of macrophages in toxic animals indicates that the usual defense mechanisms had proved inadequate, and some quantity of the colloid remained free in fluids which reached the thymic lobular spaces.

In analyzing the material presented in the preceding pages, it might be pertinent to reemphasize the findings on reaction differences between the two regions of thymic stroma. One part of this stroma is composed of the fibrous framework of the lobule, and the other of the intralobular, perivascular connective tissue. As far as could be detected, the stroma proper exhibited little or no reaction during the changes induced by involution, or during regeneration. Diminution in size of the lobules produced an apparent condensation of the fibrous framework, but there was no indication of fibrogenesis or of activation of the associated fibroblasts. If free cells of wide developmental potentiality are ever formed in this tissue, they could not be identified by the above methods.

By contrast to findings on the potentialities of stroma, the intralobular, perivascular connective tissue has retained

some degree of reactive power under conditions of this experiment; this is indicated by its rather limited ability to form phagocytes of ordinary connective tissue type. Since most of the intralobular macrophages of this type are found only in the medullary region, it is assumed that their migratory ability is limited. Indeed, their frequently stellate form would indicate that at least some of these cells are fixed within the connective tissue stroma. It appears that although derivatives of mesodermal tissues in the thymus can be identified under experimental conditions, their reactive powers are strictly limited. By far the most complex reaction seen under experimental conditions takes place in the epithelial cells, elements of entodermal origin. These cells can be stimulated to hypertrophy, to free themselves from their attachments, and to transform into thymocytes or into macrophages.

SUMMARY AND CONCLUSIONS

Two series of albino rats were injected with acid colloidal substances (Chlorazol black B or mercuric sulfide) and their thymuses examined histologically after varying periods. Animals from one of these series were injected with toxic doses of the colloid, while those of the other group were maintained as long as possible under subtoxic conditions. They were killed after various intervals in order to study the microscopic changes which appear during induced involution and in post-involutional reconstruction of the thymus.

It was found that thymuses from toxic animals undergo almost complete involution with regard to their small thymocytes, but that in every case a few of these cells escape destruction. These more resistant cells later participate in regeneration. With the advent of involution, the epithelial component of the thymus undergoes hypertrophy, and forms highly branched cords of cells whose stems lie in the medulla. Branches from the major medullary stems pass into the cortex and there terminate in bulbous expansions.

At the terminals of these epithelial cords, and to a lesser extent also along their surfaces as well, the more peripherally placed epithelial cells round up and escape into the lobular spaces. After becoming thus freed, the cells can transform into thymocytes. Throughout the process, mitotic figures are rare and even missing: early reconstruction, then, is carried out almost exclusively by a process of epithelial cell transformation. During the later stages of regeneration, mitotic figures can be found in small and medium thymocytes; presumably the dividing cells arise from the epithelium as well as from the thymocytes that escaped destruction in the involution process.

Thymuses from animals which survived several days or more after injection of the foreign colloid invariably contained loaded phagocytes. These cells are of two morphological types. One, which has been called the ordinary connective tissue macrophage, may be nearly spherical or of stellate form and typically contains numerous, small, discrete globules of the injected colloid. The other form, called the intralobular macrophage, is rounded and contains the injected material formed into complexes of highly variable size and color. The variations in shade of these segregated globules indicates that the intralobular macrophages are able to carry out some degree of digestion within the mass. This process is not completed, as indicated by persistent pale globules in the older cells. The ordinary connective tissue macrophage was found to be only weakly potent in the digestion and coalescence of ingested colloid globules.

In animals which have been initially injected with a toxic dose of the colloid substance and then allowed to recover, the thymus showed active signs of regeneration. Under these conditions, the transformation of freed epithelial cells is almost completely into new thymocytes. When toxic injections are continued, the regeneration of parenchyma is reduced in degree, and many of the epithelial cells are transformed into phagocytes. Apparently, these cells are always of the intralobular type. Ordinary connective tissue

macrophages are formed from intralobular connective tissue and from cells in the perivascular connective tissue of the thymic blood vessels. This process takes place for the most part in the thymic medulla for it is only in this region of the gland that the blood vessels are of sufficient size to possess a well formed adventitia.

LITERATURE CITED

- ANDREASEN, E., AND J. OTTENSEN 1944 Significance of the various lymphoid organs to the lymphocyte production in the albino rat. *Acta Pathol. et Microbiol. Scandinav.*, 54 (suppl.): 25-32.
- BAILLIF, R. N. 1948 Ovarian response in the albino rat to injected colloidal substances. *Am. J. Anat.*, 83: 109-142.
- DEANSLEY, R. 1928 Experimental studies on the histology of the mammalian thymus. *Quart. J. Micr. Sci.*, 72: 247-275.
- DOUTHAT, A., AND R. PABDINAS 1943 Observaciones acerca de la naturaleza y extension de los reticulos del timo. *Arch. de Histol. Norm. y Patol.*, 1: 415-439. (Cited from *Excerpta Medica*, 1948 Sect. I, 2: 512-513.)
- DUNN, T. B. 1945 Behavior of thymus tissue transplanted to a skin wound. *J. Nat. Cancer Inst.*, 5: 285-288.
- EMMART, E. W. 1936 A study of the histogenesis of the thymus in vitro. *Anat. Rec.*, 66: 59-73.
- FOULDS, L. 1934 Autoplastic transplantation of the thymus gland of the fowl. *Imp. Cancer Res. Fund, Sci. Rep. No. 11*, pp. 27-33.
- GOLDNER, J. 1925 Reaktionen der Thymus während der Knochenbrüche. *Arch. f. mikr. Anat.*, 104: 72-87.
- GOTTESMAN, J. M., AND H. L. JAFFE 1926 Studies on the histogenesis of autoplastic thymus transplantations. *J. Exp. Med.*, 43: 403-414.
- GREENE, E. C. 1935 Anatomy of the Rat. *Tr. Am Philos. Soc., New Series*, Vol. 27.
- GREGOIRE, C. 1943 Regeneration of the involuted thymus after adrenalectomy. *J. Morph.*, 72: 239-262.
- GUDERNATSCII, P. 1937 Present status of the thymus problem; review of nonsurgical experimentation in this field. *Med. Rec.*, 146: 101-109.
- HAMMAR, J. A. 1909 Fünfzig Jahre Thymusforschung. *Ergeb. d. Anat. u. Entwcklungsh., 19: 1-274* (part 1).
- 1921 The new views as to the morphology of the thymus gland and their bearing on the problem of the function of the thymus. *Endocrinol.*, 5: 543-573; 731-760.
- 1938 Data new and old on the thymus gland. *Roy. Soc. Sci., Upsala*.
- JAFFE, H. L. 1926 Autoplastic thymus transplants. II. With particular reference to the regeneration of the reticulum cells and the formation of Hassall's corpuscles. *J. Exp. Med.*, 44: 523-532.

- JONSON, A. 1909 Studien über die Thymusinvolution. Die akzidentelle Involution bei Hunger. *Arch. mikr. Anat.*, 73: 390-443.
- KINDRED, J. E. 1940 A quantitative study of the hemopoietic organs of young albino rats. *Am. J. Anat.*, 67: 99-149.
- KINGSBURY, B. F. 1941 The interpretation of thymus bodies. *Endocrinol.*, 29: 155-160.
- MATSUNAGA, M. 1910 Ueber die parenchymatösen Lymphgefäße der Thymus. *Ztschr. f. Anat. u. Entwicklgsch.*, 28: 339-348.
- MAURER, F. W. 1885-86 Schilddrüse und Thymus der Teleostiere. *Morph. Jahrb.*, 11: 129-175.
- MAXIMOW, A. A. 1909 Untersuchungen über Blut und Bindegewebe. II. Über die Histogenese der Thymus bei Säugetieren. *Arch. f. mikr. Anat.*, 74: 525-621.
- 1912a Untersuchungen über Blut und Bindegewebe. IV. Über die Histogenese der Thymus bei Amphibien. *Arch. f. mikr. Anat.*, 79: 560-611.
- 1912b Untersuchungen über Blut und Bindegewebe. V. Über die embryonale Entwicklung der Thymus bei Selachiern. *Arch. f. mikr. Anat.*, 80 (part 1): 39-88.
- MICHEL, N. A. 1938 The mast cells in *Handbook of Hematology*. Hal Downey, Ed. Vol. I, Sect. IV, Paul B. Hoeber, Inc., New York.
- MURRAY, R. G. 1947 Pure cultures of rabbit thymus epithelium. *Am. J. Anat.*, 81: 369-411.
- NELSON, W. O. 1939 Relation of the thymus and pineal glands to genital function. Chapter 21 in *Sex and Internal Secretions*. Edgar Allen, Ed., Williams and Wilkins, Baltimore.
- PAPPENHEIMER, A. M. 1910 A contribution to the normal and pathological histology of the thymus gland. *J. Med. Res.*, 17: 1-75.
- 1913 Further studies of the histology of the thymus. *Am. J. Anat.*, 14: 299-332.
- POPOFF, N. 1926 The histogenesis of the thymus as shown by tissue cultures, transplantation and regeneration. *Proc. Soc. Exp. Biol. and Med.*, 24: 148-151.
- 1927 The histogenesis of the thymus as shown by tissue cultures. *Arch. f. exp. Zellforsch.*, 4: 395-418.
- ROSS, M. A., AND V. KORENCHIEVSKY 1941 The thymus of the rat and sex hormones. *J. Path. and Bact.*, 52: 347-360.
- RUDBERG, H. 1907 Studien über die Thymusinvolution. I. Die Involution nach Röntgenbestrahlung. *Arch. Anat. u. Entwicklgsch. Anat. abtlg. (suppl.)*: 123-174.
- SÆLYE, H. 1936 Thymus and adrenals in the response of the organism to injuries and intoxications. *Brit. J. Exp. Path.*, 17: 234-248.
- SMITH, C. 1946 Some histological changes in the thymus of the rat during age involution. *Anat. Rec.*, 96: 580. (Abstract.)
- STÖHR, P. 1906 Ueber die Nature der Thymus-Elemente. *Anat. Hefte*, 31: 407-455.

- TSCHASSOWNIKOW, N. 1926 Über die *in vitro*-Kultures des Thymus. Arch. f. exp. Zellforsch., 2: 250-276.
- WASSEN, A. L. 1915 Beobachtungen an Thymuskulturen *in vitro*. Anat. Hefte, 52: 279-318.

PLATE I

EXPLANATION OF FIGURES

All figures are unretouched photomicrographs of specimens fixed in Bouin or Zenker's fluid, sectioned at 5μ and stained with Harris' hematoxylin and eosin.

1 A thymic lobule at the beginning of involution; from an animal injected with a single toxic dose of Chlorazol black E and sacrificed 24 hours later. A small portion of the medulla is here seen at the upper left, and the perilobular connective tissue at the lower right. The destructive process is indicated by pyknosis of thymocyte nuclei and by collection of thymocytes into groups; this grouping is particularly noticeable in the cortex. $\times 367$.

2 Margin of a thymic lobule at the height of the destructive process. This specimen was taken from an animal subjected to a single toxic dose of HgS and killed before recovery could be effected. Especially near the superficial surface of the lobule (at the bottom of the figure) extensive thymocyte destruction is in progress; hypertrophied nuclei of the epithelium can be seen scattered among the pyknotic thymocyte nuclei. A single acidophilic cell, marked by an arrow, lies in the perilobular connective tissue. $\times 743$.

3 Perilobular connective tissue from the thymic region of an animal injected with repeated toxic doses of Chlorazol black E. Note the numerous heavily loaded macrophages that lie adjacent to the lobular capsule, which is at the lower right corner of the figure. The great majority of these macrophages are rounded in form and appear to be lying free in the tissue spaces, but a few are stellate and are anchored in the connective tissue stroma. Occasional thymocytes and acidophilic cells appear among these macrophages. $\times 743$.

4 A fully involuted thymic lobule from an animal that had received repeated toxic injections of Chlorazol black E. The upper left portion of the figure is filled with medulla and cortex of the lobule, the intermediate portion with the lobular capsule, and at the lower right is the surface of the gland. Note the open character of cortex and medulla and the presence of epithelial cells and intralobular phagocytes in the tissue spaces. Note also the fibrous nature of the capsule and particularly at its outer surface, the presence of numerous macrophages of ordinary connective tissue type. $\times 367$.

5 The periphery of a regenerating thymic lobule, taken from the gland of an animal which had received a single toxic injection of Chlorazol black E and was sacrificed on the third post-injection day. The cortex, now filled with many newly formed thymocytes, occupies the upper portion of the figure. Some of the thymocytes are infiltrating the thick capsular region which fills the remainder of the figure. In this region are also colloid laden macrophages of ordinary connective tissue type and several partially degenerated acidophilic cells. $\times 743$.

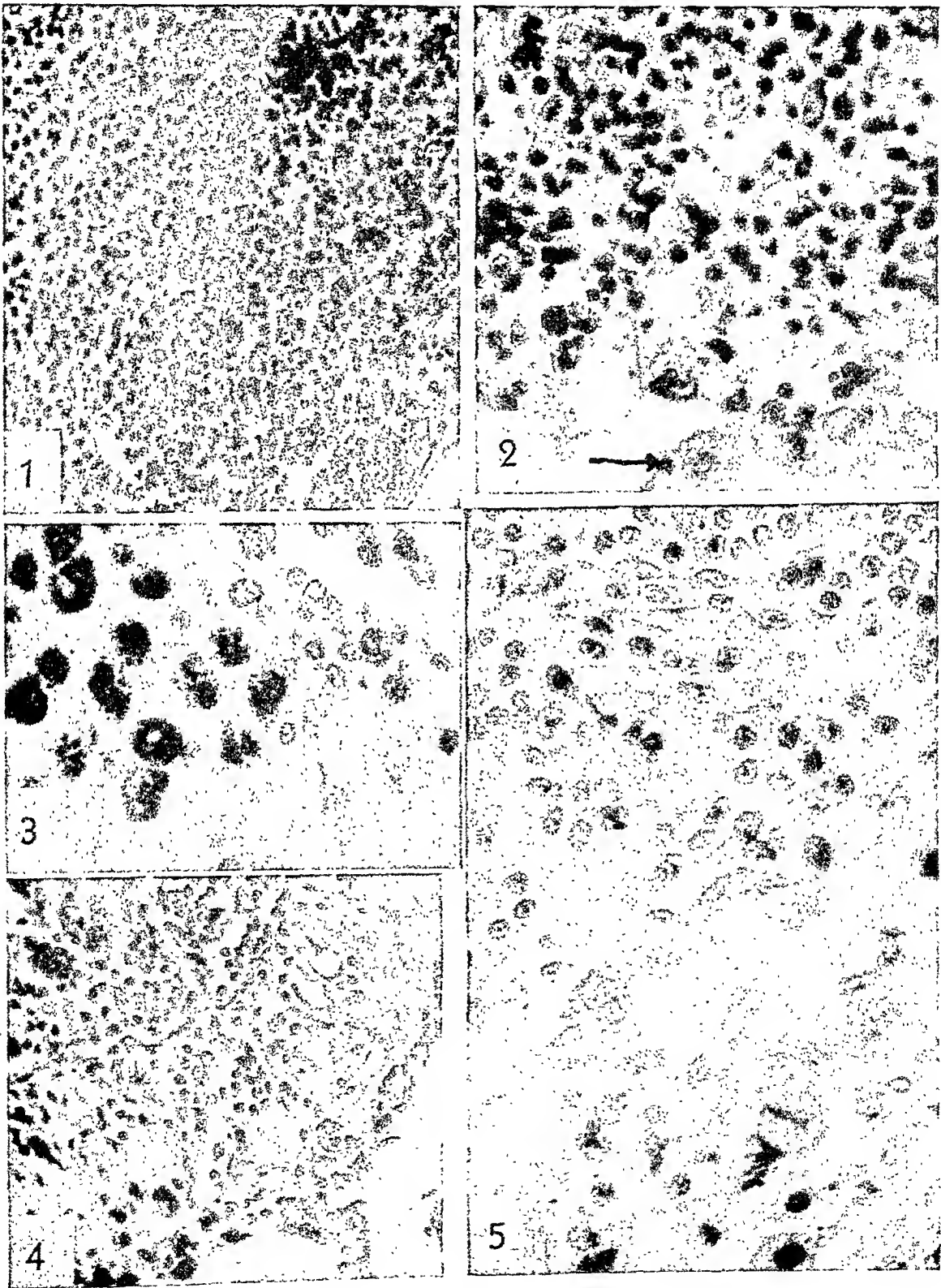


PLATE 2

EXPLANATION OF FIGURES

6 Periphery of a lobule from a thymus that was regenerating after a single toxic injection of Chlorazol black E. The cortex appears at the right of the figure and the capsule fills the remainder of the figure. At this time, the capsule is richly infiltrated with thymocytes. At the center of the figure and indicated by the arrow, is a spray of epithelial cells from which individual elements appear to be separating and transforming into new thymocytes. Several intralobular macrophages appear near the right side of the figure; the capsule contains occasional acidophilic cells. $\times 743$.

7 The capsule of a thymic lobule at the height of regeneration; this specimen was taken from an animal which had been injected with a single toxic dose of Chlorazol black E, three days previously. The figure shows dense infiltration of the fibrous network by thymocytes that have migrated from the subjacent cortex. The rounded nuclei with chromatin aggregated mainly in strands are those of hypertrophic fibroblasts; the associated fibrous network is clearly evident. The densely staining nuclei are those of invading thymocytes. $\times 743$.

8 An acidophilic cell from the interlobular connective tissue of a thymus at the advent of regeneration; this animal was sacrificed two days after a single toxic dose of Chlorazol black E. Note the rounded form of this cell and its nuclear pattern, similar to that of the thymocyte. In the histological preparation, the densely stained cytoplasm is brilliantly acidophilic; in many cases, cytoplasm of such cells appears granular, but when the granules are numerous and large (as in the instance shown here) they appear to undergo fusion. $\times 1428$.

9 Interlobular connective tissue showing macrophages that are loaded with the injected colloid (Chlorazol black E); this animal received repeated toxic injections and was sacrificed following the third dose. Note that the colloid globules are collected in multiple foci within the cytoplasm and that these foci tend to remain discrete. The macrophages shown here are stellate in form and appear to be still attached to the associated tissue framework (cf. fig. 3). Whenever nuclei of these cells are unobscured by the collected colloid, they exhibit a chromatin pattern identical with that typical of the ordinary histiocytes of the region. $\times 1428$.

10 Small portion of a thymic lobule taken from an animal sacrificed when *in extremis* following repeated toxic injections of HgS. Note the almost complete absence of thymocytes and the relatively small degree of hyperplasia in the epithelium. The most significant feature of this lobule is the occurrence of many macrophages; a large intralobular macrophage is seen at the top of the figure, and several of ordinary connective tissue type appear near the bottom of the figure. $\times 743$.

11 Early regeneration within a thymic lobule; this specimen was secured from an animal that had begun to recover from a single toxic dose of Chlorazol black E. A strand of hypertrophic epithelium crosses the figure from upper right to lower left; it is such strands that form the epithelial cords shown in the following illustrations. Note that the nuclei of these cells are of variable size and form, and that they are marked internally by scattered strands of chromatin. The thymocytes shown here are degenerating as indicated by the pyknosis of their nuclei and by wrinkling of their cell margins. $\times 743$.

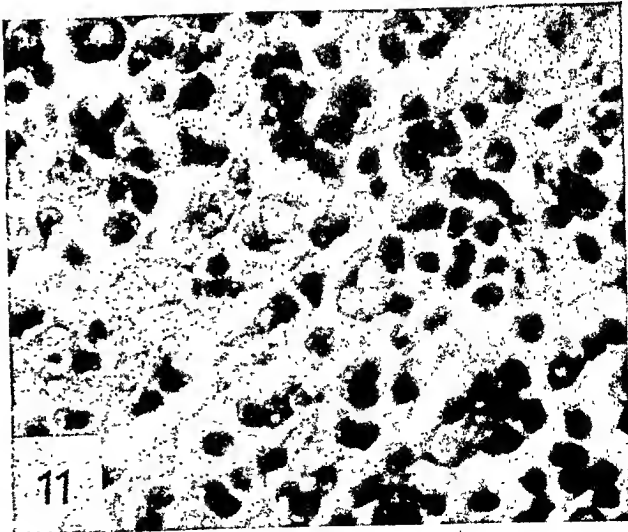
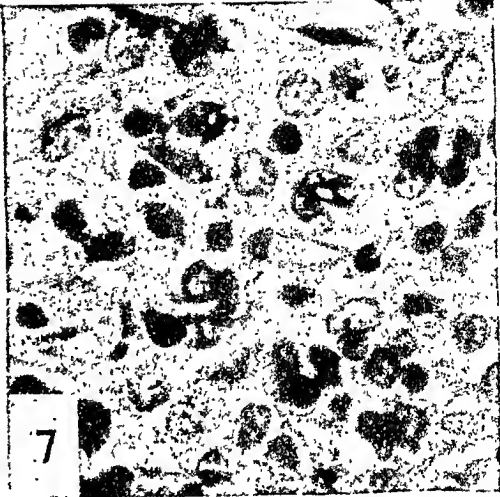
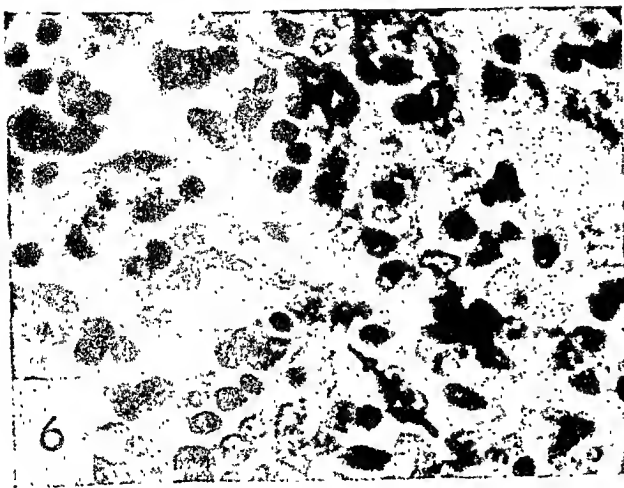


PLATE 3

EXPLANATION OF FIGURES

12 A forming epithelial cord, similar to that seen in the preceding figure, is here shown in cross section at the right. This thymus was taken from an animal 48 hours after a single toxic dose of Chlorazol black E. There is some indication that cells are being freed from the periphery of such cords, even during their formation. Note that the nuclei of adventitial cells of the vessel immediately to the left of the epithelial cord in this figure do not exhibit the same chromatin pattern as do those of epithelium. The adventitial cells (supposedly of mesodermal origin) have nuclei which are typically smaller in size, more flattened in form, and with greater concentration of chromatin, as compared with those of the epithelium (thought to be of entodermal origin). $\times 743$.

13 Thymic regeneration during repeated subtoxic injections of Chlorazol black E. This figure shows the striking contrast in structure between thymic parenchyma and epithelium during lobular regeneration. A thick cord of epithelial cells extends from the upper left downward and toward the lower edge of the figure. This cord terminates in several rather bulbous branches, some of which are hollow. The margins of the cord and its terminal branches are marked by many cells that are pulling free from the reticulum and undergoing transformation into thymocytes. This latter process is shown in greater detail in the following figures. $\times 367$.

14 Thymic lobule in regeneration after repeated subtoxic injections of Chlorazol black E. This illustration presents a longitudinal section of an epithelial cord similar to that shown in figure 13. Note that the margins of the cord are regionally separated from the main mass to form spaces in which lie partially or wholly freed cells. Such cells show all stages of the separation process, and of transformation from epithelial to thymocyte type. $\times 743$.

15 A small portion of an epithelial cord from a thymus during regeneration following repeated subtoxic injections of Chlorazol black E. Two groups of epithelial cells are shown, each formed into a small follicle. Note the pale-staining nuclei with chromatin distributed primarily in strands; some of these nuclei contain prominent nucleoli. $\times 1428$.

16 Early thymic reaction to repeated subtoxic doses of Chlorazol black E. The epithelial cells that form a group (in the medulla of a lobule) are beginning to enlarge and to form vacuoles in which the foreign colloid is collected. Thymocytes, shown at the right of the figure, are readily identified by their small, darkly staining nuclei. These are sharply contrasted with the larger, lightly staining nuclei of epithelial elements from which the phagocytes are being formed. $\times 1428$.

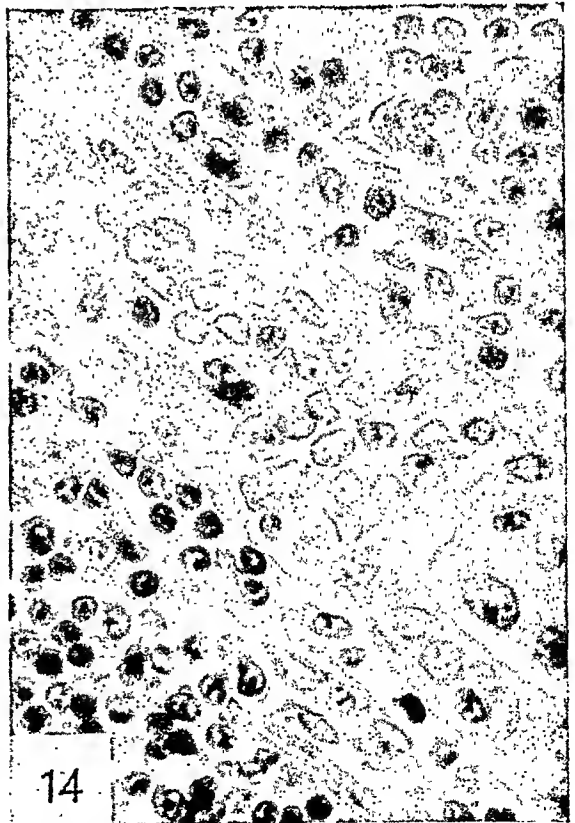
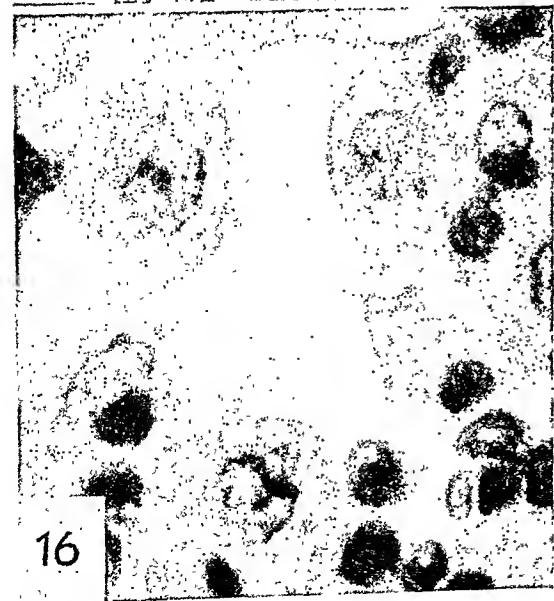
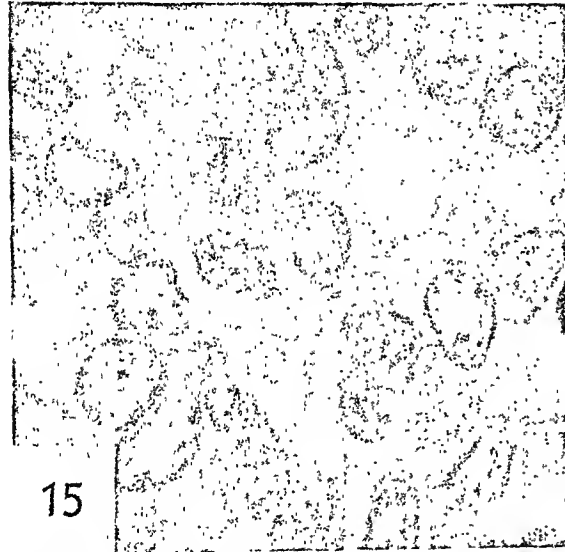
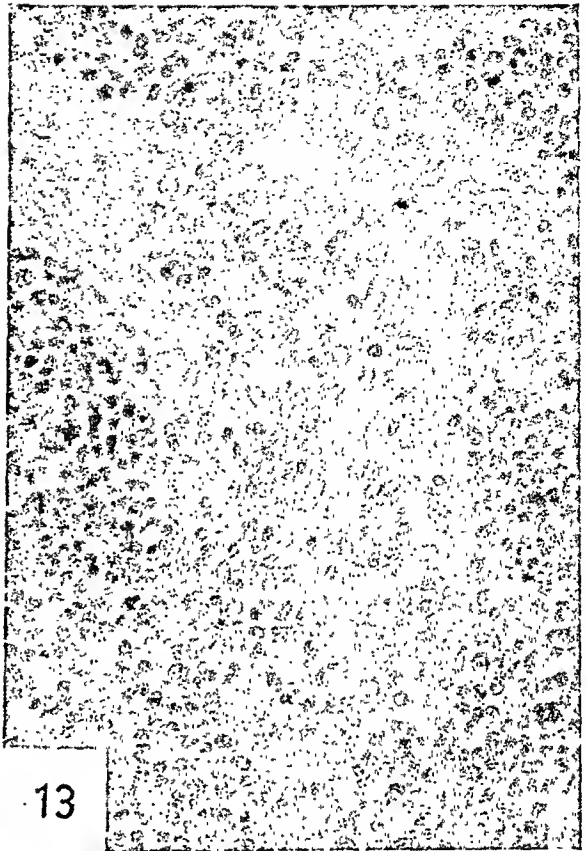
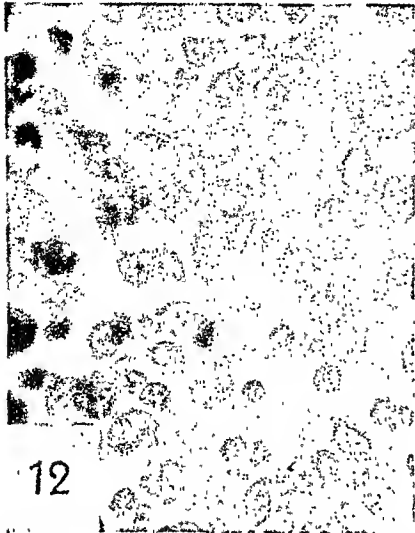


PLATE 4

EXPLANATION OF FIGURES

17 The periphery of a lobule in early regeneration following a single toxic injection of HgS. Note the preponderance of epithelial cells with nuclei in which there is a relative paucity of stainable chromatin; many of these nuclei exhibit prominent nucleoli. Some reticular cells are transforming into intralobular phagocytes that are collecting globules of the foreign colloid. These globules are already in the process of coalescence into complex masses of variable sizes. $\times 743$.

18 A lobule from a thymus in full regeneration after a single toxic injection of Chlorazol black E. The upper left corner of the figure is occupied by medulla, and the perilobular connective tissue appears at the lower border; the intervening area is composed of cortex. The medulla is packed with thymocytes that probably arose from ancestors which escaped the initial destructive process. The cortex is filled with scattered thymocytes and with intralobular phagocytes that have nearly completed digestion of the contained colloid material. Several acidophilic cells appear in the perilobular connective tissue. $\times 367$.

19 Early giant cell formation from fusion of intralobular macrophages, in a thymus regenerating after a single toxic injection of HgS. This complex appears to be composed of one element that contains the two nuclei at the lower right, a second element containing the single nucleus at the upper left, and a third part at the lower left, containing a single nucleus and several incompletely digested colloid masses. $\times 1428$.

20 Later stage of giant cell formation from intralobular macrophages in a thymus regenerating after a single toxic injection of HgS. Note that no intercellular boundaries can be detected even at this magnification, although vacuoles with digesting colloid are readily visible. This giant cell contains 5 nuclei and presumably arose from fusion of as many macrophages. $\times 1428$.

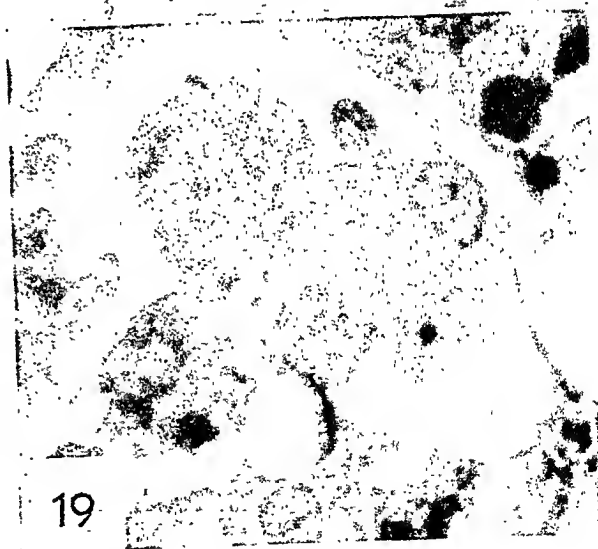
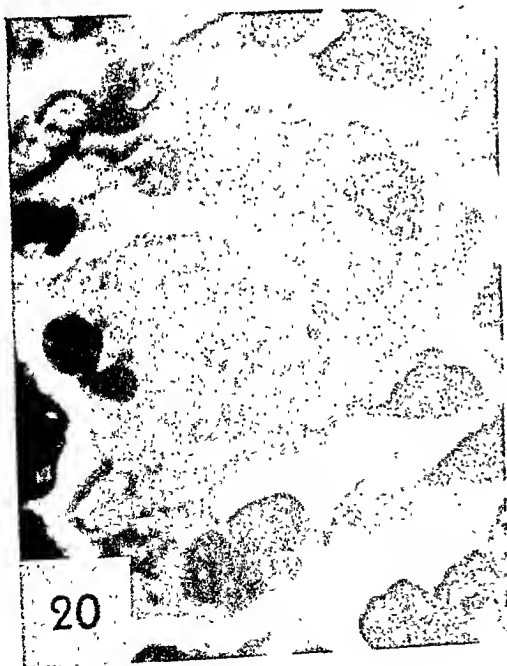
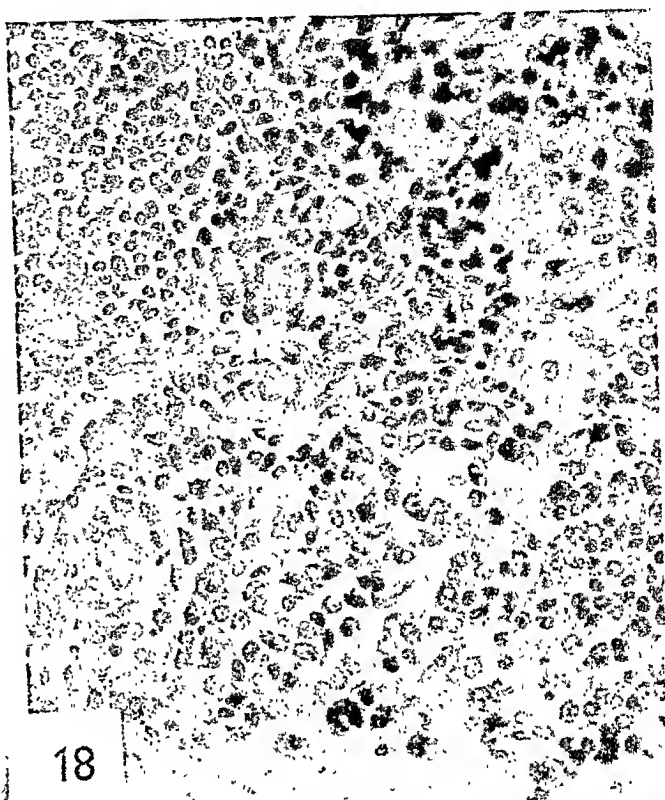
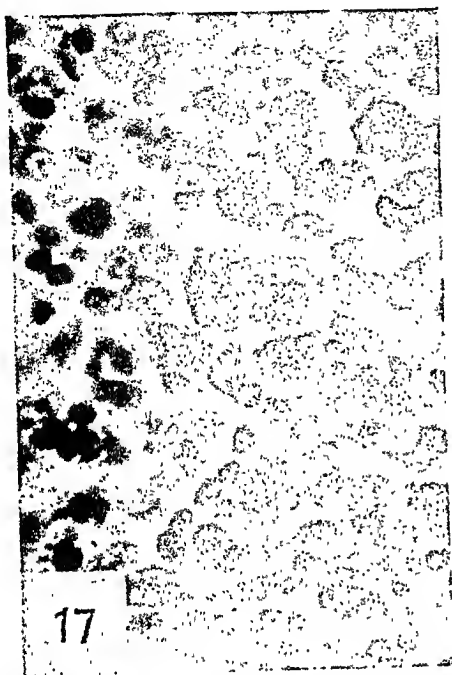


PLATE 5

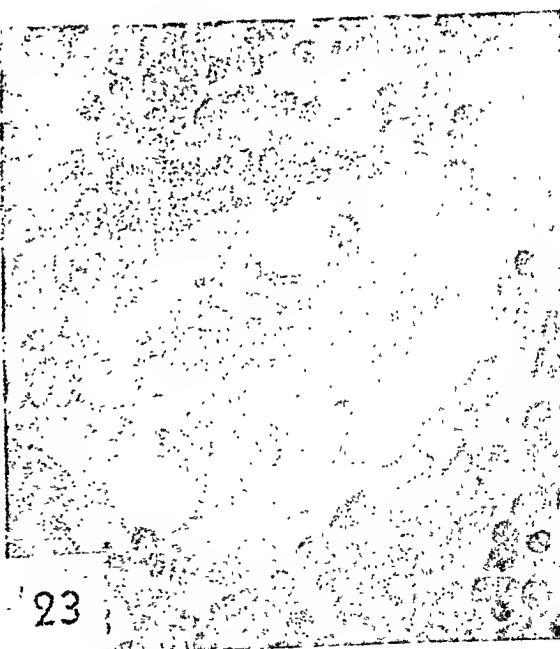
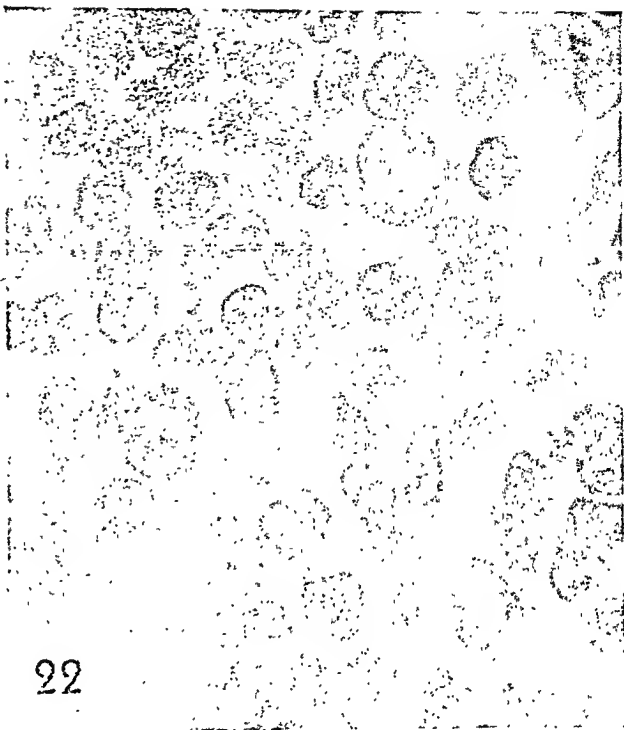
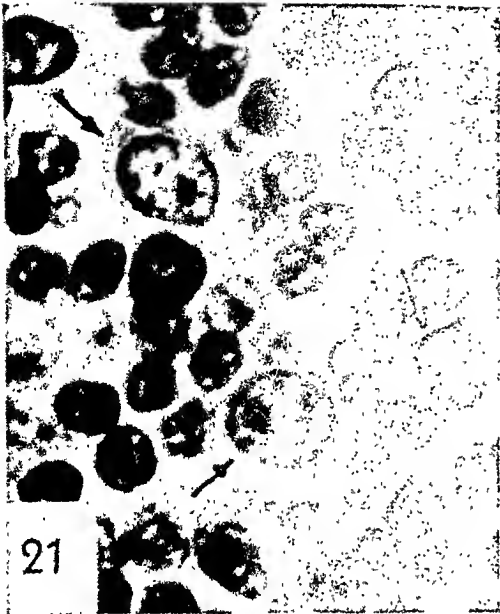
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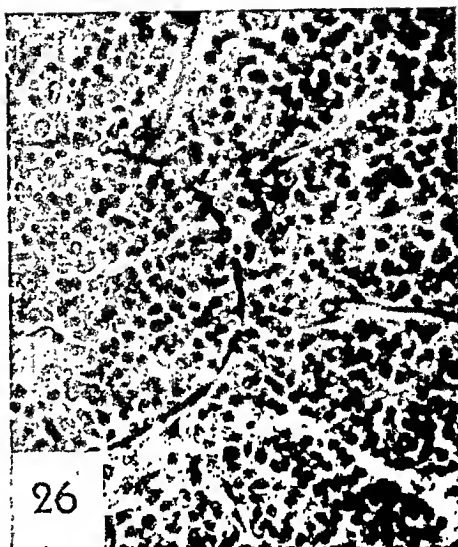
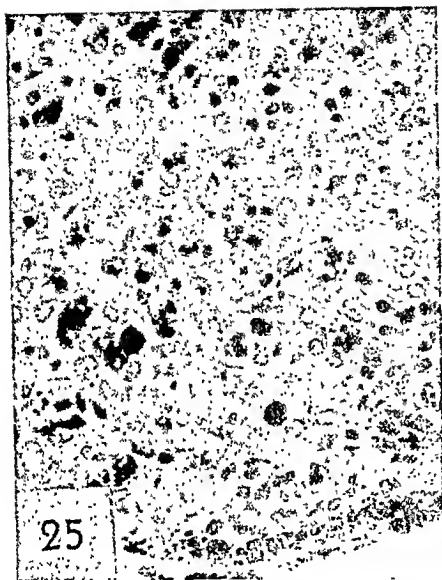
21 The margin of an epithelial cord from the thymus of an animal recovering from a single toxic injection of Chlorazol black E. A segment of the epithelium appears in the central portion of the figure, and from it three cells marked by arrows, are in the process of separation. The distinctive nuclear pattern of cells in the epithelium is still retained despite the fact that these nuclei have rounded up and greatly increased in size. Numerous thymocytes lie in the meshes of the epithelial reticulum. $\times 1428$.

22 Another region from the specimen shown in the preceding figure. An epithelial cord appears at the lower and left margins of the figure. Several cells are pulling free from the network and a single element, shown in the upper central region, has completed the separation process. This latter cell has many of the cytological characteristics of the primitive lymphocytogenic stem cell. $\times 1428$.

23 A colloid-filled follicle in the medulla of a thymic lobule. The content of this follicle is distinctly acidophilic; several disintegrating cells are lodged within it. Note that vacuoles in the colloid lie adjacent to well defined cuboidal cells, while its denser portions adjoin the more squamous elements. $\times 743$.

24 Profound cellular destruction in the thymus after repeated toxic doses of HgS; animal sacrificed when *in extremis*. Fibroblasts of the lobular capsule and some epithelial cells still retain their normal cytologic characteristics, but all freed elements are in the process of destruction. $\times 743$.





25 Thymic lobule from an animal subjected to repeated toxic injections of HgS and sacrificed when *in extremis*. This is an actively regenerating gland, with many phagocytes both of the ordinary connective tissue and intralobular types. Note that thymocytes are rarely seen; the lobule appears to be composed of a hypertrophic epithelium whose freed cells are phagocytic in character. $\times 367$.

26 Late regeneration of the thymic lobule of an animal allowed to recover after a single toxic injection of Chlorazol black E. The boundary between cortex and medulla is indicated by the broken line. In this specimen few phagocytes remain. Thymocytes are numerous in both cortex and medulla, and the epithelial elements are becoming compressed. It is now difficult to differentiate the epithelium from the connective tissue stroma of the lobule. $\times 367$.

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